Regulation of maize *Ac/Ds* transposition by replication and DNA methylation

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ABBREVIATIONS

A	Adenine
ACMV	African Cassava Mosaic Virus
Amp	Ampicillin
ATP	adenosine 5’-triphosphate
bp	base pairs
BSA	bovine serum albumine
C	Cytosine
^{5m}dCTP	5-methyldeoxycytidine 5'-triphosphate
CT-DNA	calf thymus DNA
CTP	cytosine 5’-triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease I
dsDNA	double stranded DNA
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
Exp.	Experiment
Fig.	Figure
G	Guanine
GUS	β-glucuronidase
hr	hour(s)
HMG	high mobility group
kb	kilobase
kDa	kilo Dalton
iae	increased *Ac* excision
IR	intergenic region
IHF	integration host factor
IRBP	inverted repeat binding protein
LB	Luria broth
LTR	long terminal repeat
LUC	Luciferase
min	minute(s)
m.o.i.	multiplicity of infection
n.d.	not determined
NLS	nuclear localization signal
nu.	nucleotide
ori	origin of replication
ORF	open reading frame
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEG	polyethyleneglycol
Prom.	promoter
Rep	Replicase
RIP	Repeat-Induced Point mutation
RNA	ribonucleic acid
RT	room temperature
rpm	rotations per minute
sec	second(s)
SAM	S-adenosylmethionine
SAP	Shrimp Alkaline Phosphatase
SDS	sodium dodecylsulfate
ssDNA	single stranded DNA
ST-DNA	salmon sperm DNA
T	Thymine
Tab.	Table
TGMV	Tomato Golden Mosaic Virus
TIR	terminal inverted repeat
TPase	Transposase
Tris	Trishydroxymethyl-aminomethan
TSDs	target sites duplications
TYLCV	Tomato Yellow Leaf Curl Virus
u	units
UTR	untranslated terminal region
UV	ultra violet
WDV	Weat Dwarf Virus
X-Gluc	5-bromo-4-chloro-3-indolyl-β-glucuronid

SUMMARY

In maize the transposable elements *Activator*/*Dissociation* (*Ac*/*Ds*) transpose shortly after replication from one of the two resulting chromatids ("chromatid selectivity"). A model was suggested that explains this phenomenon as a consequence of different *Ac* transposase binding to holo-, hemi- and unmethylated transposon ends (Wang *et al*., 1996). It assumes that before replication the element is holomethylated and does not transpose because TPase can not bind to the transposon ends. Shortly after replication one of the two differentially hemimethylated daughter transposons should become transposition competent.

However, DNA methylation-mediated replication dependence does not completely explain the behavior of *Ac*/*Ds* transposition. In several studies performed in monocot hosts, it was found that in a transient assay *Ac*/*Ds* element excision from extrachromosomal geminivirus vectors occurs only during vector replication, although the transfected DNAs were not C-methylated.

In this work the correlation between *Ds* transposition, DNA replication and DNA methylation in the dicot species *Petunia hybrida* was studied. *Ds* reporter vectors harboring TYLCV (Tomato Yellow Leaf Curl Virus) geminivirus replicon sequences and replicating in transfected petunia cells were constructed. It has been shown that the transposition of a *Ds* element from an extrachromosomal vector in petunia cells is regulated by DNA replication in a methylation-dependent and -independent mode. Holomethylation completely inhibits *Ds* excision from a non-replicating plasmid, whereas *Ds* transposition is restored by replication. Moreover, *Ds* elements that are hemimethylated on one DNA strand transpose in the absence of replication, whereas methylation on the complementary DNA strand results in at least 6.3-fold reduced excision frequencies. These data strongly support the transposition model of Wang *et al*. (1996).
Beyond that, *Ds* transposition is strongly promoted by replication also in the absence of methylation. It has been shown that in petunia cells, unlike monocot hosts, replication is not a prerequisite for *Ds* transposition, nevertheless it enhances *Ds* transposition by at least a factor of 7.5. Moreover, replication promotes the formation of a predominant excision footprint. Implications on the mechanism and regulation of *Ac*/*Ds* transposition are discussed.

1 INTRODUCTION

The controlling elements of maize were the first transposable elements discovered. The systematic genetic analysis of mutations in maize caused by transposable elements began with the work of Emerson (Emerson, 1914; Emerson, 1917; Emerson, 1929) and Rhoades (Rhoades, 1936; Rhoades, 1938; Rhoades, 1941; Rhoades, 1945) on unstable mutations affecting pigment biosynthesis. But it was the elegant studies of McClintock (McClintock, 1948; McClintock, 1949) on elements belonging to the *Ac*/*Ds* family that in 1948 provided the first evidence that genetic elements transpose and that unstable mutations could result from the insertion of a transposable element. McClintock first identified the *Ds* element as a specific site of chromosome breakage or *Dissociation* (McClintock, 1946). Chromosome breakage at *Ds* did not occur spontaneously, but required the presence of a second genetic element, designated the *Activator* (*Ac*), for its ability to activate chromosome breakage at *Ds* (McClintock, 1947). Observations that *Ac* insertion alleles of a locus can give rise directly to *Ds*-like mutations at the same locus suggested that *Ds* and *Ac* elements were structurally related.

The vast majority of active transposons uncovered in plants to date fall into the two superfamilies of "hAT" and "CACTA" elements. Members of each family have common characteristics at the element termini, the transposase (TPase) protein, and the target site duplications. *Ac* belongs to the "hAT" (*hobo*/*Ac*/*Tam3*) elements superfamily that includes in addition to *Ac*/*Ds* elements, the *Tam3* element of *Antirrhinum*, as well as a number of related elements from other plants and from invertebrates (such as *hobo* transposon of *Drosophila*).

1.1 *Ac* and *Ds* DNA structure

1.1.1 The *Ac* element

The first *Ac* element was isolated from the *waxy* (*wx*) locus of maize, which encodes an abundant starch-biosynthetic enzyme (Fedoroff *et al.*, 1983; Shure *et al.*, 1983). Much progress has since been made on the structural characterization of the element.

Ac is a 4565 bp transposable element that consists of a *trans*- and a *cis*-acting region (Fig. 1).

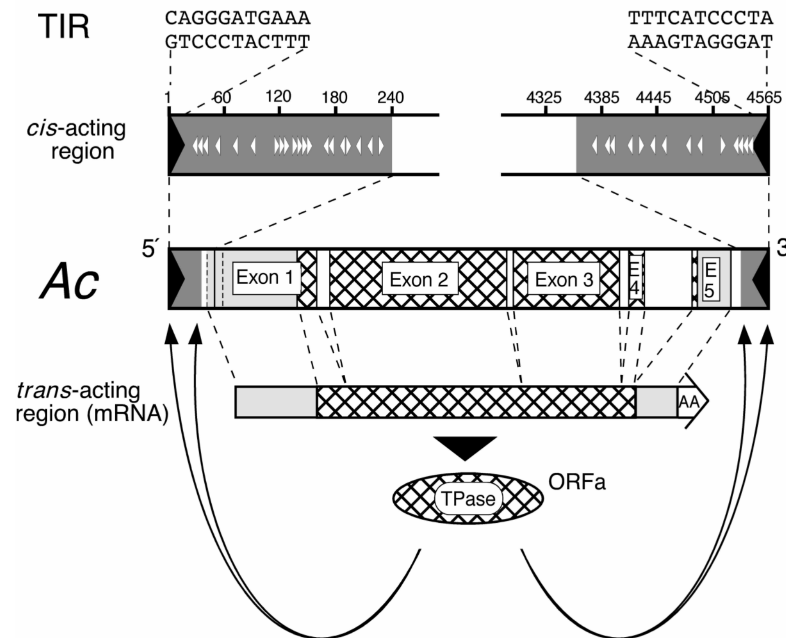


Fig. 1. Structure of the *Ac* element. The 4565 bp *Ac* element consists of *trans*- and *cis*-acting regions. The *trans* acting region encodes the TPase protein. Transcription is initiated at multiple sites between positions 280 and 380 and the 3.5 kb mRNA consists of 5 exons. ORFa is the 2421-nucleotide-long TPase open reading frame. The *cis*-acting regions correspond, at both termini of *Ac*, to 11 bp imperfect terminal inverted repeats (TIRs), whose sequences are shown in the upper line, and subterminal regions. The distribution of AAACGGs and closely related motifs, the TPase binding sites, within the subterminal regions is indicated by small arrow-heads. TPase binding to the TIRs and to the subterminal regions catalyses the transposition process.

1.1.1.1 *Trans*-acting region

The *trans*-acting region of the *Ac* element, corresponding to transposon internal sequences, encodes a single 807 amino acid polypeptide, the transposase protein (TPase), which is necessary and sufficient to mobilize *Ac* and non-autonomous *Ds* elements (Kunze *et al*., 1987; Coupland *et al*., 1988). The single transcription unit extends over most of the element's length (starting around 300 nucleotides from the 5' end and terminating 264 nucleotides from the 3' end) and encodes a 3.5 kb mRNA comprising 5 exons and encoding a 2421 nucleotide-long TPase open reading frame (ORFa). Several sites of transcription initiation have been identified between positions 280 and 380 from the 5' end

of the element. The major initiation site is at position 334 (Kunze *et al.*, 1987; Finnegan *et al.*, 1988); accordingly, the *Ac* promoter region overlaps the TPase binding sites region. The *Ac* promoter is lacking a CAAT and a TATA box and therefore is reminiscent of mammalian housekeeping gene promoters (Dynan, 1986). Like these, the *Ac* promoter is weak and appears to be constitutively active. Polyadenylation takes place 265 bp proximal to the 3' end (Kunze *et al.*, 1987; Finnegan *et al.*, 1988).

Through a bipartite DNA binding domain (Becker and Kunze, 1997), the *Ac* TPase binds to the TIRs and, with greater affinity, to repetitive subterminal binding sites (see below) in the *Ac* ends and catalyses the transposition reaction. Binding to the subterminal motifs is accomplished by a C-terminal subdomain alone, whereas recognition of the TIRs also requires a N-terminal sub-domain. The binding appears to occur in a cooperative mode by interactions between TPase molecules (Becker and Kunze, 1997); this would stabilize the protein-DNA transposition complex that is supposedly formed as intermediate structure during the transposition reaction. Such "self-interaction" functions of the TPase are localized at the C-terminus of the protein (Boehm *et al.*, 1995; Essers *et al.*, 2000).

1.1.1.2 *Cis*-acting region

The *cis*-determinants for *Ac* transposition are located at both ends of the element and consist of 11 bp terminal inverted repeats (TIRs), whose outermost nucleotides are not complementary, plus approximately 240 bp subterminal regions that contain the TPase target sequences (Coupland *et al.*, 1988; Coupland *et al.*, 1989; Varagona and Wessler, 1990) (Fig. 2). Both TIRs and subterminal sequences define the members of the *Ac/Ds* transposable family and are obligatory for transposition. A replacement of the four terminal bases of the 3'-TIR of *Ac* (Hehl and Baker, 1989) or of the five terminal bases of the 5'-TIR (Healy *et al.*, 1993) as well as the replacement of the 11 bp *Ac*-TIRs by the related 12 bp TIRs from the *Tam3* element (Chatterjee and Starlinger, 1995) immobilizes the element completely. Internal deletions at either end of *Ac* result in a gradual decrease in excision frequency: the element is immobilized when 116 bp or less at the 5' end or less than 102 bp at the 3' end are retained (Coupland *et al.*, 1988; Coupland *et al.*, 1989).

The subterminal regions are very G+C rich (45% and 40% of the sequence composition respectively at the 5' and 3' ends of the element), and there is a strong bias toward the arrangement of these two bases in potential cytosine methylation sites such as CpG or

CpNpG (Gruenbaum *et al.*, 1981). The 5' and 3' ends contain 26 and 24 CpG dinucleotides respectively but only one GpC sequence each (Kunze *et al.*, 1988). In contrast, the G+C content of the untranslated leader region is 68%, without any bias in CpG *versus* GpC distribution. The coding region contains 38% G+C and CpG sequences are under-represented.

Many of the CpG potential methylation sequences found at both *Ac* ends are part of the TPase binding sites found in multiple copies and orientations throughout the 5' and 3' end subterminal regions (Kunze and Starlinger, 1989; Feldmar and Kunze, 1991; Becker and Kunze, 1997) (Fig. 2). The minimal *Ac* TPase binding sites are trinucleotides with the sequence ACG or TCG, of which 25 copies are found in the 5' end and 20 copies in the 3' subterminal region; however, TPase affinity is highest when these sites are flanked on the 3' side by an additional G residue (A/TCGG), which is found in 75% of the binding sites. Most prominent are TPase binding sites in the repetitive AAACGG motifs.

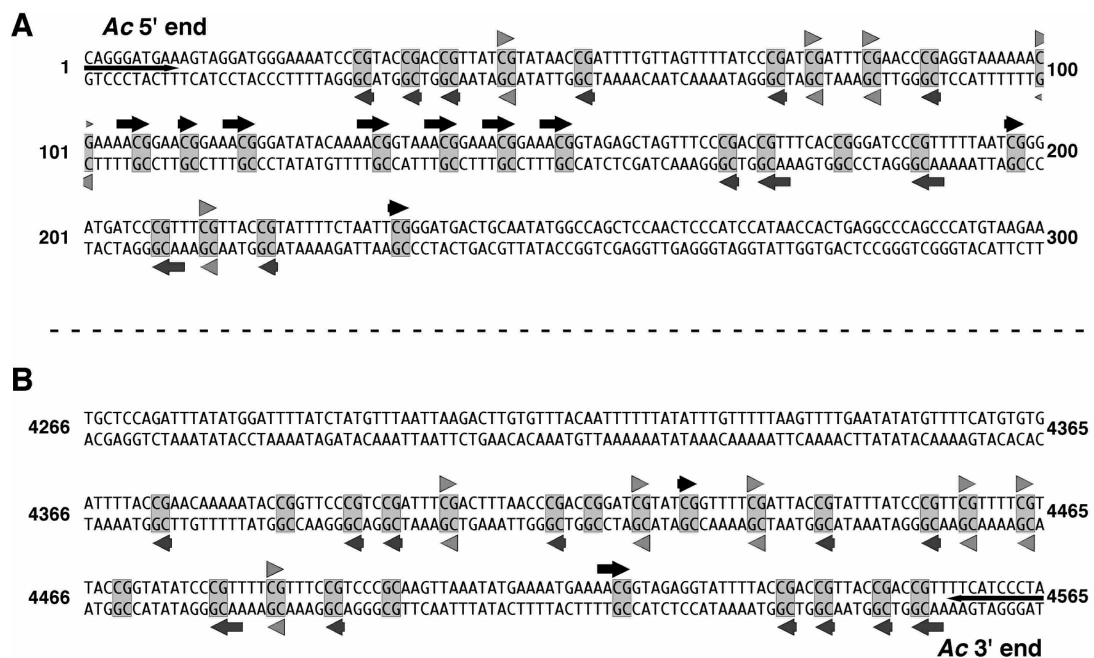


Fig. 2. Potential TPase binding sites in the subterminal regions of *Ac*. A) *Ac* 3' end. **B)** *Ac* 5' end. The terminal inverted repeats are indicated by the black arrows between the DNA strands. CpG dinucleotides are highlighted by shaded boxes across both DNA strands. *Lightly shaded arrowheads* above and below the DNA strands indicate ACG and TCG motifs. *Short arrows* are depicted in correspondence of ACGG and TCGG sequences, and *long arrows* show AAACGG sequences. TPase binding sites oriented toward the 5' inverted repeat are indicated by *grey arrows* and those oriented toward the 3' inverted repeat are indicated with *black arrows*.

In *Ac* and *Ac*-derived *Ds* elements, the AAACGG and A/TCGG motifs are involved in the transposition reaction, as mutations of individual copies or groups of binding sites in the element cause a reduction in excision frequency (Chatterjee and Starlinger, 1995).
Importantly the A/TCGG and AAACGG TPase binding sites are not randomly oriented at both ends of *Ac* (see Fig. 2 and schematically depicted in Fig. 22A and C). At the 5' end, the six sites between residues 28 and 90 are oriented toward the inverted repeat, whereas the following seven sites between residues 103 and 152 have the opposite orientation. Five of the seven innermost sites between positions 166 and 235 are also oriented toward the end of the element. The situation at the 3' end of *Ac* is even more biased: of the fourteen TPase binding sites with defined orientation, twelve are oriented toward the center of the element. The G+C characteristic composition and the structured distribution and orientation of the TPase target sites within the element have important implications for the regulation of *Ac*/*Ds* transposition.

1.1.2 The *Ds* elements

Ds elements are the non-autonomous members of the *Ac*/*Ds* maize transposon family (McClintock, 1948). They have proved surprisingly varied in structure, but what they importantly share are essential sequences, such as the TIRs and the TPase binding sites, required in *cis* for transposition, whereas the *trans*-acting transposase is inactivated. These elements arise from the autonomous *Ac* element by deletions and other changes of internal sequences. The mechanism by which these elements originate is still under investigation, but the repair synthesis pathways acting on the donor site after an *Ac* element excised appears to play an important role (Yan *et al*., 1999).

1.2 Mechanism of the transposition reaction

Transposition of *Ac*/*Ds*, as in general for maize DNA transposable elements, occurs conservatively by a "cut and paste", non replicative mechanism, and is accompanied by its disappearance from the donor location and reintegration into a new site of the genome.

1.2.1 Transposase/DNA complex formation: the "transpososom" complex

How *Ac* TPase catalyses the transposition reaction is still under investigation. An essential requirement for a "cut and paste" mechanism of transposition is healing of the chromosome breaks that are generated during excision, thereby avoiding chromosomal loss. Joining of the breaks is probably brought through close physical proximity of the transposon ends. An hypothetical model for the *Ac* transposition complex, the transpososom, has been proposed (Kunze *et al*., 1997). Accordingly, two functions have been postulated, one that is required for the association of the two ends of *Ac* and one that cuts close to the ends of the element. It is possible that both functions are carried out by the *Ac* TPase itself. The TPase binds to the subterminal sites at both ends of the element, and by interactions between TPase molecules the two ends are aligned. The catalysis of strand cleavage by transposase involves nicking of the DNA to generate nucleophilic 3' OH groups on both strands. 5' ends are also cleaved in the synaptic complex, releasing the transposable element from the donor DNA.

The synaptic complex possibly encompasses the transposition target site. Through target DNA capture, the target DNA becomes bound to the synaptic complex, followed by strand transfer, where the 3' OH groups of the transposable element perform a nucleophilic attack on both strands of the target DNA. The attack of 3' OH groups on the target DNA occurs with a staggered spacing between insertion sites that is specific for each transposable element. For *Ac*/*Ds* this spacing is 8 base pairs. The resulting target site duplications (TSDs) are not required for the excision process (Dooner *et al*., 1988; Lechelt *et al*., 1989; Peterson, 1990). It is still unknown whether host factors are involved that facilitate the transpososom complex assembly.

1.2.2 Footprint formation and models for transposon excision mechanism

Ac/*Ds* elements can influence gene expression as well as alter genome structure in a variety of plant hosts, and therefore act as important "natural" mutagens. The excision of *Ac*/*Ds* elements can cause chromosome breaks, inversions and translocations; however, the most frequent results of *Ac*/*Ds* excision are small sequence alterations, or "transposon footprints" left at the donor site. Usually in maize as well as in many heterologous plants where *Ac* actively transposes the TSDs are retained upon excision, but excision frequently is imprecise and associated with small or large deletions of the adjacent sequences or with

small insertions of a few bases (Pohlman *et al*., 1984; Sutton *et al*., 1984; Weck *et al*., 1984). Whether gene function is restored for transposons inserted within exons depends on the number and sequence of bases added or removed (Wessler, 1988). The original target sequence is restored only rarely (Baran *et al*., 1992; Scott *et al*., 1996), but presumably this is the result of a TPase mediated reciprocal recombination process between the TSDs and not of transposition.

Depending on the insertion allele, footprints vary in sequence and in the number of bases from the original target duplication that are left behind. Usually one or two predominant footprints are formed for each allele, displaying either deletion, inversion or replacement by their complement of the one or two base pairs closest to the element (Scott *et al*., 1996; Rinehart *et al*., 1997). Less frequent footprints at each site appear similar to the predominant type, often having additional mutations progressing outward from the center of the original target sequence. Deletions that extend beyond the TSDs are rare.

How do these footprints originate?

After examining mutations left by well-characterized insertions and then attempting to reconstruct the events surrounding element excision, two models for footprint formation by end-joining have been proposed (Saedler and Nevers, 1985; Coen *et al*., 1989) (Fig. 3) that differ with respect to the main enzymatical events during the excision.

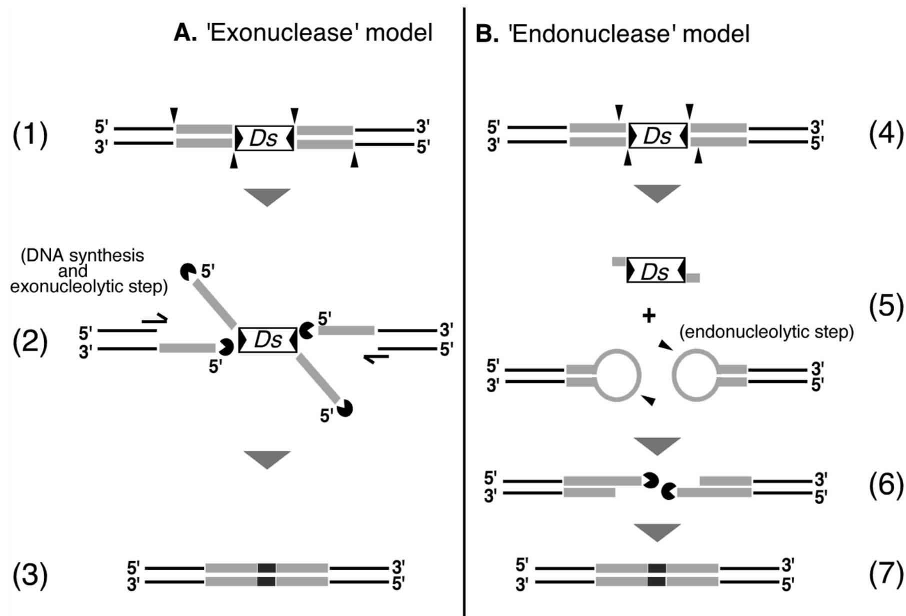


Fig. 3. Schematic of the models for *Ac* footprints formation. A) 'Exonuclease' model **B**) 'Endonuclease' model.

These models have been recently revised by Scott *et al*. (1996) and Rinehart *et al*. (1997), based on a more representative and unbiased view of excision products in individual plants by using the polymerase chain reaction to examine footprints formed in somatic tissues. According to the "exonuclease model" of Saedler and collegues (1985) (Fig. 3A), the initial cleavage by TPase (precise target duplication staggered incisions) (1), is followed by a combination of DNA repair fill-in synthesis and 5' to 3' exonuclease degradation on the single strand overhangs protruding from the flanking DNA and from the element itself (2). The element is then released and the two chromosomal ends religated and mismatches repaired (3). On the other hand, the "endonuclease model" of Coen *et al*. (1989) (Fig. 3B), suggests that after 1-bp-staggered cuts generated by the TPase at the transposon ends (4), the element is immediately removed and the DNA ends left behind close on themselves to form hairpin loops (5). An endonuclease opens these loops and the released ends are ligated to each other and mismatches repaired (7).

1.3 Regulation of *Ac/Ds* transposition

Transposable elements can be very dangerous for the host if their activity is not controlled somehow. Several mechanisms have been developed during the "co-evolution" of the transposon/host system that regulate the timing and the frequency of transposition.
In maize, the *Ac* element transposes usually only in a fraction of the cells, but the transposition frequencies vary over a wide range. The germinal excision frequencies of *Ac* or *Ds* at different loci may vary in the range of 0.01% to more than 15%; at the *bz2::Ds2* locus in the aleurone cells *Ds2* transposes with frequencies of 0.2-1% (reviewed in Kunze, 1996). *Ac/Ds* transposition is regulated at different levels that explain these low and variable transposition frequencies. Some mechanisms are transposon-specific whereas others involve rather plant-specific factors.

1.3.1 Chromosomal context

Ac elements at different genomic positions differ in excision timing and frequency and give rise to different excision patterns by the same *Ds* element (McClintock, 1963; McClintock, 1964; Schwartz, 1984; Schwartz, 1986). It is likely that the chromosomal context modulates the expression of the TPase in respect to the level and/or the time of synthesis and consequently the excision patterns of the *Ac* element itself and of *Ds*

elements mobilized *in trans* (Balcells and Coupland, 1994; Heinlein, 1995). Either the promoter of *Ac* interacts with enhancers of the gene promoter where the element is inserted or epigenetic differences between the elements (such as DNA C-methylation) are involved.

1.3.2 *Ac/Ds* dosage effects

Activator normally displays a negative dosage effect in maize. An increase in *Ac* copy number in the endosperm from one to two or three leads to a developmental delay of transposition events and a decrease in transposition frequency. As a result of this inverse dosage effect, maize kernels show less and smaller revertant sectors (McClintock, 1948; McClintock, 1951). After further analyses of reversion sectors from different *Ac* alleles it became clear that depending on the *Ac* allele, the dosage effect can either be inverse or positive, and for a particular allele the dosage effect can change from negative to positive during endosperm development (Heinlein and Starlinger, 1991).

In heterologous systems, *Ac* normally displays a positive dosage effect; increasing copies of *Ac* result in earlier and more frequent excisions of somatic and germinal *Ds* excision (Jones *et al*., 1989; Yoder, 1990; Swinburne *et al*., 1992). However, when *Ac* transposase is expressed from a strong constitutive promoter, transposition is inhibited (Scofield *et al*., 1992; Swinburne *et al*., 1992; Scofield *et al*., 1993).

To explain this behaviour of *Ac/Ds*, it has been proposed that the dosage effect depends on the *Ac* TPase protein concentration in the nucleus and its spatial or temporal distribution. Inhibition of *Ac* or *Ds* excision occurs when the TPase concentration increases beyond a certain threshold level. When and where this threshold can be reached for a given allele appears, at least in maize, to be under the control of host cellular signals (Schwartz, 1986; Kunze *et al*., 1988; Brutnell *et al*., 1997).

The negative dose effect is presumably effected by the formation of inactive TPase aggregates (Heinlein *et al*., 1994).

1.3.3 *Ac/Ds* transposition is associated with replication and displays chromatid selectivity

Some aspects of the mechanism and the regulation of *Ac* and *Ds* transposition are not yet fully understood. One central question concerns the role of DNA replication.

Many elegant genetics and molecular studies performed between the beginning of the sixties and the beginning of the nineties (Greenblatt and Brink, 1962; Greenblatt, 1968; Greenblatt, 1974; Greenblatt, 1984; Chen *et al*., 1987; Chen *et al*., 1992) culminated in the conclusion that *Ac* transposes predominantly during or shortly after the S phase of the cell cycle and that after replication only one of the two daughter elements is transposition competent. Most of this information came from the analysis of *Ac* transposition at the *P* locus from maize. The *P-RR* allele of the *P* locus conditions the red pigmentation of the cob and pericarp. An insertion of *Ac* in this allele (which gives rise to the *P-VV:E* allele) suppresses *P* function and causes a colorless pericarp phenotype. *Ac* excision restores *P-RR* gene action and somatic red sectors of different size arise, causing a variegated pericarp phenotype known as medium variegated. The presence of a second active *Ac* element in addition to the *P-VV:E* allele due to inverse dosage effect causes a reduction in pericarp striping to a phenotype of few red stripes, known as light-variegated pericarp.

On medium-variegated ears, early transpositions of *Ac* result in large and contiguous sectors overlying multiple kernels of red and light-variegated pericarp approximately 80% of the time and are referred to as "twin mutations" (or "twin sectors") (Greenblatt, 1974).

Genetic and molecular analyses of twin mutations at the donor and target sites before and after *Ac* insertion have shown that co-twin sectors are derived from a single transposition event of *Ac* occurring from only one of the daughter elements during or shortly after replication (Greenblatt and Brink, 1962; Chen *et al*., 1987; Fedoroff, 1989; Chen *et al*., 1992). Twin sectors are of two types, type I and type II, based respectively on the presence or absence of *Ac* activity in the red twinned sector (Fig. 4). Both red twinned sectors have no *Ac* at the donor *P-VV* site; at the target site *Ac* is present only in type I red twinned sectors.

Light variegated sectors in both types of twin mutations carry an active *Ac* at both the donor *P-VV* site and the target site.

Moreover, in type I twin sectors the *Ac* target site is identical in both daughter chromosomes.

The most plausible explanation that is offered to explain the occurence of such twinned sectors is that *Ac* is excised after replication from one *P-VV* chromatid and reinserted either into an unreplicated target site (type I twin formation, Fig. 4a) or into an already replicated target site on the sister chromatid (interchromatid transposition) (type II twin formation, Fig. 4b). This implies that the enzyme required for excision of *Ac* is not active on the

unreplicated transposon and differentiates between the two daughter elements after replication (chromatid selectivity).

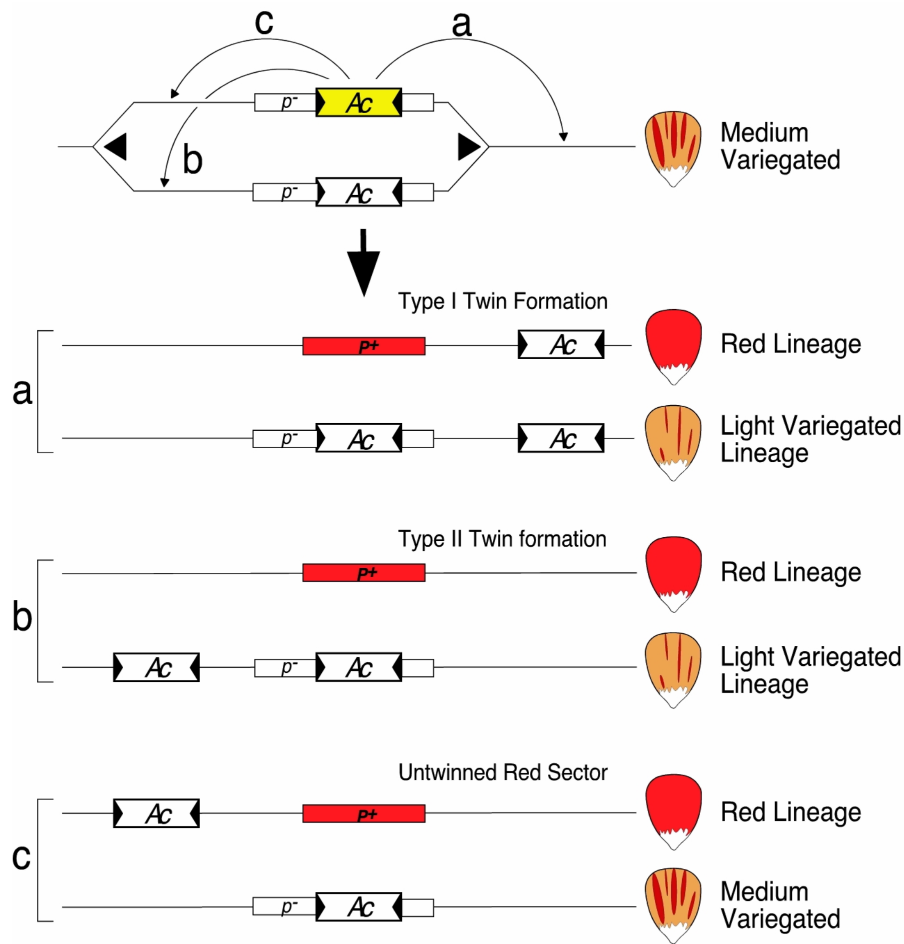


Fig. 4. *Ac* transposes after replication. Genotypes and phenotypes resulting from transposition of *Ac* from the *P* gene. Maize kernels carrying one *Ac* element are medium variegated. After replication, only one of the two daughter *Ac* elements is transposition competent ('chromatid selectivity'). Reinsertion into replicated and unreplicated DNA results in three genetically different pairs of daughter cells. Insertion into unreplicated DNA **(a)** results in a revertant (phenotypically wild type) daughter cell carrying one *Ac*, and the other carrying two *Ac* elements. Due to the 'inverse dosage effect' of *Ac*, kernels derived from cells with two *Ac*s are lightly variegated. Reinsertion into replicated DNA **(b)** can result in a *Ac*-free revertant daughter cell line and a cell line carrying two elements, giving rise to lightly variegated kernels. Alternatively **(c)**, reinsertion into replicated DNA can result in both daughter cell lines carrying one *Ac*. The resulting kernels are revertant and medium variegated.

The fact that most early transposition events during ear morphogenesis are twinned suggests that replication of *Ac* may be a prerequisite for transposition. However the presence of untwinned sectors of red and light variegated pericarp in medium variegated

ears (Greenblatt, 1974), whose generation mechanism is still unclear, rises the possibility that the dependence of transposition on replication is not so tight.
Only few of the observed untwinned sectors can be readily explained with the same mechanism that yields twin mutations. This is the case for untwinned red sectors (Fig. 4c) for instance, if we assume that transposition occurs from a replicating donor site to a target site on the same chromatid. The remaining untwinned events are possibly the product of a yet unclear transposition mechanism, not necessarily associated with replication.

1.3.4 Epigenetic regulation of transposition: the role of DNA CpG methylation

The characteristics of the *Ac* transposition reaction and its dependence on replication are similar to those of the bacterial *IS10* element; in this case transposition is regulated by DNA adenine methylation of the *IS10* TPase binding sites. Normally these sites are methylated and the element is inactive. After passage of the replication fork, two transiently hemimethylated *IS* elements are formed. One hemimethylated *IS10* is more active than the other and at least 1000-fold more active than the fully methylated element *in vivo* (Roberts *et al.*, 1985).
This nice example provided by the prokaryotic kingdom gave rise to the question whether DNA methylation could represent the factor linking transposition to replication for *Ac* as well.

1.3.4.1 DNA binding of *Ac* TPase is methylation sensitive

A significant fraction of the potential methylation sites (CpG and CpNpG) in the ends of *Ac* are part of the TPase binding sites. One relevant feature of TPase binding sites is that they are non palindromic DNA sequences. *In vitro* studies have shown that the affinity of the TPase to its target motifs increases in comparison with the unmethylated target site if one or both cytosine residues on the one strand (TTTGCC) are replaced by 5-methylcytosine (Tab. 1). In contrast, DNA binding is totally inhibited by C-methylation on the opposite strand (AAACGG), irrespective of whether the cytosine residues on the complementary strand are methylated or not (Kunze and Starlinger, 1989; Kunze *et al.*, 1991). This was the first evidence of the asymmetric nature of the TPase-DNA interaction and suggested that methylation of TPase binding sites is of functional importance for transposition.

Tab. 1. Binding efficiency of the *Ac* TPase to differentially C-methylated target sites. The TPase target DNAs consisted of 18 tandem copies of the indicated hexamer motifs. The relative binding efficiency of *Ac* TPase was determined by gel-shift assay (Kunze *et al.*, 1991). ^{m}C, 5-methyl-cytosine. (+), very weak binding that can be obscured by increasing the competitor-DNA concentration.

TPase target sites	TPase binding efficiency
AAACGG TTTGCC	+
AAACGG TTTG^{m}CC	++
AAACGG TTTGC^{m}C	++
AAACGG TTTG^{m}C^{m}C	+++
AAA^{m}CGG TTTGCC	–
AAA^{m}CGG TTTG^{m}CC	–
AAA^{m}CGG TTTGC^{m}C	–
AAA^{m}CGG TTTG^{m}C^{m}C	(+)

1.3.4.2 The TPase binding sites within *Ac* are methylated *in vivo* prior to replication

In maize kernels, the subterminal regions of *Ac* and *Ds* elements are heavily C-methylated. This was first investigated by using methylation sensitive enzymes (Schwartz and Dennis, 1986; Schwartz, 1989), but it became particularly clear after genomic sequencing of the active *Ac* (*Ac9*) in the *wx-m9::Ac* allele and of the inactive *Ac* (*Ds-cy*) (Schwartz and Dennis, 1986) in the *wx-m9::Ds-cy* allele of maize (Wang *et al.*, 1996; Wang and Kunze, 1998). Most of the C-methylation was found to be restricted to the cytosines of the TPase binding sites at both ends of the element.

All of the TPase target sites were hypermethylated at the transposon 3' ends in both alleles. CpG sequences were almost 100% methylated in both alleles; a more variable pattern of C-methylation was observed for CpNpG sequences.

At the 5' end, the situation was different: in *Ds-cy* all TPase target sites were heavily methylated, whereas in *Ac9* cytosine methylation was either completely absent or restricted to the TPase binding sites between residues 28 and 90 (as in the case at the 3' end, C-

methylation showed a bias toward CpG than CpNpG sequences and non-symmetrical C residues in both alleles).

Because the 5' end contains the *Ac* promoter overlapping with the TPase binding sites between residues 100 and 235, and because *Ac9* actively produces transposase, it is conceivable that transcription factors bound to this region interfere with methyltransferase activity by steric hindrance. This is probably why in *Ac9*, C-methylation at the 5' end is so underrepresented (Wang *et al*., 1996).

In *Ds-cy*, hypermethylation of the promoter region has been correlated with the loss of element-encoded transposase gene expression and inactivity of the element (Schwartz and Dennis, 1986; Chomet *et al*., 1987; Kunze *et al*., 1988; Schwartz, 1989; Brutnell and Dellaporta, 1994). However, *Ds-cy* can be mobilized by an active *Ac* element present in the genome (Schwartz and Dennis, 1986; Kunze *et al*., 1988).

1.3.4.3 Model for chromatid-selective activation of *Ac/Ds* following replication

Based on the results described in the previous sections, a model has been proposed to explain the association with replication and the strand selectivity of *Ac* transposition (Wang *et al*., 1996; Wang and Kunze, 1998) (Fig. 5).

Before replication, the 5' end of *Ds-cy* and the 3' end of both elements (Fig. 5A and 5B) are unable to bind TPase. In *Ac9* the 5' end is either completely or only at the most internal binding sites occupied by the TPase (in Fig. 5A is illustrated the situation by partially methylated 5' end). Both elements should be stable. Immediately after replication, previously methylated recognition sites become hemimethylated. Because these sites occur in both orientations in either end of *Ac*, both ends of each pair of daughter elements can now be bound by TPase. However, the arrangement of TPase proteins differs at the 3' end of the daughter transposons and also at the 5' end of those *Ac* elements that were partially or totally methylated before replication.

At the 3' end, 12 of the 14 sequence motifs that strongly bind TPase *in vitro*, have the same orientation (Becker and Kunze, 1997). As a consequence, in one daughter element, in particular the one hemimethylated on the upper strand, the hemimethylation pattern of these 12 sites will favour TPase binding, whereas the other one has only two, rather distant sites whose hemimethylation pattern would allow TPase binding.

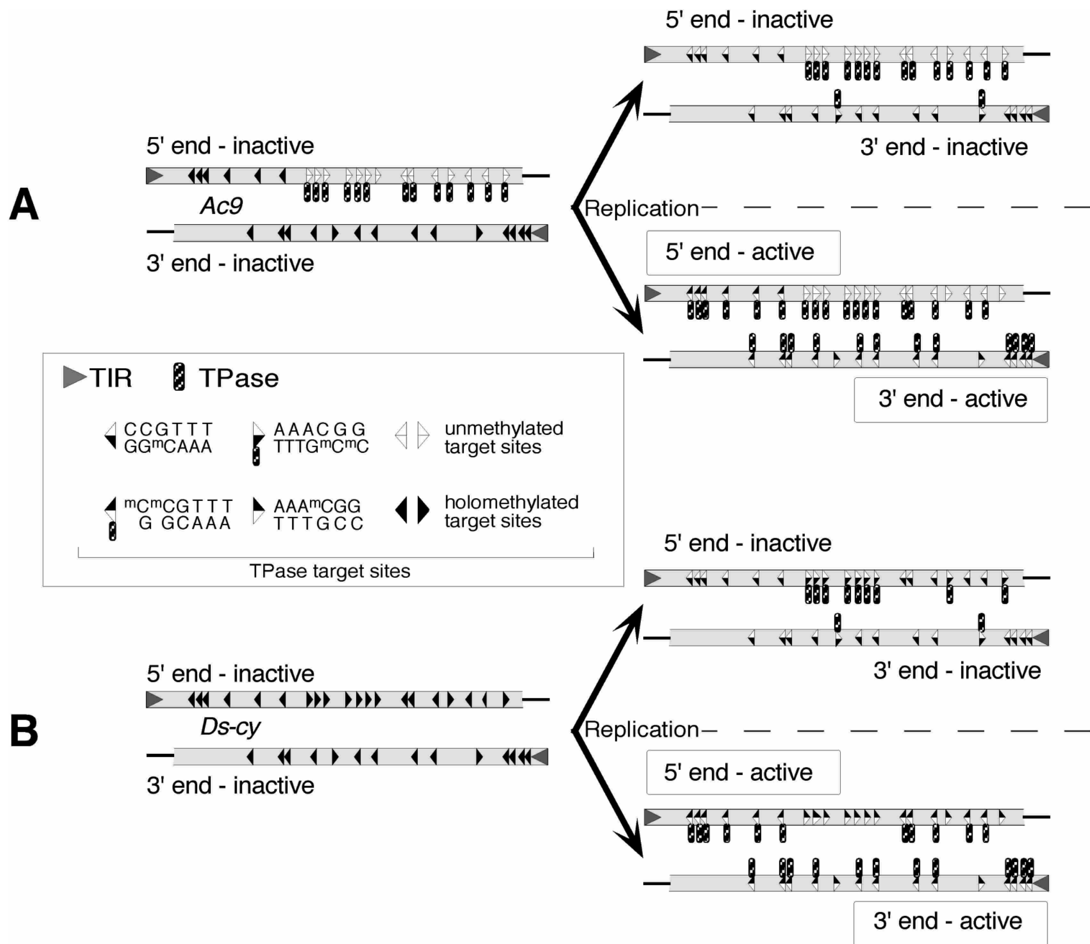


Fig. 5. Model for the 'chromatid selectivity' of *Ac* transposition. A) Prior to replication, TPase binding sites at 3' subterminus are methylated in *Ac9* and not recognized by TPase. The 5' end is only partially (at the most internal binding sites) or completely (not illustrated in the Figure) recognized by the TPase. The extremely inefficient occupation of the 3' end by the TPase should render the element stable. After replication, the two daughter elements are hemimethylated on opposite DNA strands. As a result of the differential occupation patterns of the TPase at the 3' end (and only partially at the 5' end) of each daughter element, it is proposed that only one daughter element is transposition competent. **B)** Prior to replication, in *Ds-cy* all the TPase binding sites at both ends are fully methylated and hence unable to bind TPase. The element should be stable. After replication, the differential occupation patterns of the TPase at the 3' end, with the contribution, in this case, of the 5' end of each daughter element should occur for the transposition competence of only one daughter element.

At the 5' end, the hemimethylation pattern that should account for a higher occupation of the 3' end by the TPase, would favour the binding of the protein to the terminal and innermost groups of target sites in *Ds-cy*; in *Ac9* the total 5' end would be recognized.
The opposite pattern of methylation favors the binding of the TPase to the central and most internal clusters of binding motifs in *Ac9* and only to the central cluster in *Ds-cy*. The

hypothesis is that only the newly replicated daughter element carrying methylation on the upper strand and thus binding TPase at the very 5' and 3' ends is competent for transposition, whereas the sister element is not.
Actually in this model, methylation of the 5' end does not play the major role in regulating the "*cis*" transpositional competence, as it is instead the case for the 3' end. Rather, the 5' end methylation patterns of *Ac9* and *Ds-cy* elements reflect their transcriptional activity.

1.3.5 DNA methylation does not completely explain the replication dependence of *Ac/Ds* transposition

Several studies have shown that in transient transfection assays, *Ac/Ds* element excision from extrachromosomal geminivirus vectors in maize, barley, wheat, and rice cells is tightly dependent on vector replication, although C-unmethylated DNAs were used for transfection. Sequences provided by the geminivirus Wheat Dwarf Virus (WDV) and necessary for the replication of the virus in monocot hosts were inserted in *Ac/Ds* reporter plasmids. These were consequently able to replicate in transfected protoplasts. After *Ds* excision analysis, it was observed that *Ds* actively transposed only from replicating plasmids, whereas in the absence of replication (using completely deleted or non functional replicon sequences) no *Ds* excision could be detected (Laufs *et al*., 1990; McElroy *et al*., 1997; Wirtz *et al*., 1997).
This evidence indicates that, in addition to DNA methylation, other mechanisms are probably also involved that explain the dependence of *Ac/Ds* transposition on replication. But these findings apparently conflict with the results of other studies performed in dicots such as petunia, parsley, and *Nicotiana plumbaginifolia* cells (Houba-Hérin *et al*., 1990; Becker *et al*., 1992; Kunze *et al*., 1993; Heinlein *et al*., 1994; Houba-Hérin *et al*., 1994a; Houba-Hérin *et al*., 1994b; Chatterjee and Starlinger, 1995; R. Lütticke and R. Kunze, unpublished). In these works *Ds* was shown to actively excise, in transiently transfected protoplasts, from extrachromosomal reporter vectors that actually did not carry any specific function to replicate in eukaryotic cells. Nevertheless the inability of these reporter plasmids to replicate in transfected protoplasts was never proved, therefore it can not undoubtedly be assessed that in dicots *Ds* excises independently on replication.

1.4 Aim of the work

The purpose of the experiments carried out in this work, was to develop an *in vivo* experimental system to:

a) investigate in dicot plants the regulation of *Ac*/*Ds* transposition by DNA replication in a methylation-independent mode. More data are needed to clarify the real behaviour of *Ac* in dicots in a 'replication' and 'non-replication' context. Only then can it be assessed whether, in absence of DNA C-methylation, replication affects *Ac*/*Ds* transposition in a monocot- and dicot-specific way.

b) to challenge *in vivo* the model proposed by Wang and collegues for the chromatid-selective activation of *Ac*/*Ds* following replication: can DNA methylation modulate the transposition competence of the element before and after replication?

2 RESULTS

This work purposes to study the transposition competence of the *Ds* transposon in dependence on replication, to understand and compare monocot and dicot host species in the way this dependence occurs, and to follow the effects of replication on *Ds* transposition in association with DNA methylation. Fundamentally, three experimental conditions were indispensable to this research: first of all, an *in vivo Ds* excision assay needed to be established in order to allow the investigation and accurate comparison of the frequencies with which the *Ds* element can transpose in different situations; second, a dicot cell system was needed in order to perform these *in vivo* studies and third, a "replicating" *Ds* element was needed that could be easily distinguished from a "non-replicating" *Ds* element.
The first part of the results section will describe the development of the appropriate experimental system in which all the conditions above illustrated are contemplated; the second and third part describe how this experimental system was employed to study how the dependence of *Ds* transposition on DNA replication is regulated in dicots, in the absence (the second part) and in the presence (the third part) of DNA C-methylation.

2.1 Establishment of an *in vivo* experimental system to study the behaviour of a replicating and non-replicating *Ds* element (independently or in association with DNA C-methylation)

2.1.1 Petunia protoplast transfection system

The excision properties of the *Ds* transposon were studied *in vivo* in a dicot cell system, consisting of the *in vivo* petunia (*Petunia hybrida* ssp. RL01 X ssp. Blue) protoplast transfection assay that was developed by Houba-Hérin and colleagues (Houba-Hérin *et al.*, 1990). This assay allows the investigation and comparison of the frequencies with which the *Ds* element can transpose from each donor plasmid used.
The priciples of this *in vivo* experimental system are schematically depicted in Fig. 6.
Shoot cultures of *Petunia hybrida* were grown under sterile conditions at 26°C under a 16 hr light/8 hr dark cycle and protoplasts were isolated from mesophyll of 3-5 weeks old shoots. Aliquots of approximately 10^6 protoplasts were co-transfected with a *Ds* excision

reporter plasmid and a TPase expression vector. Transfection of protoplasts was performed with use of polyethylenglycol (PEG). After transfection, the protoplasts were cultured in a rich medium.

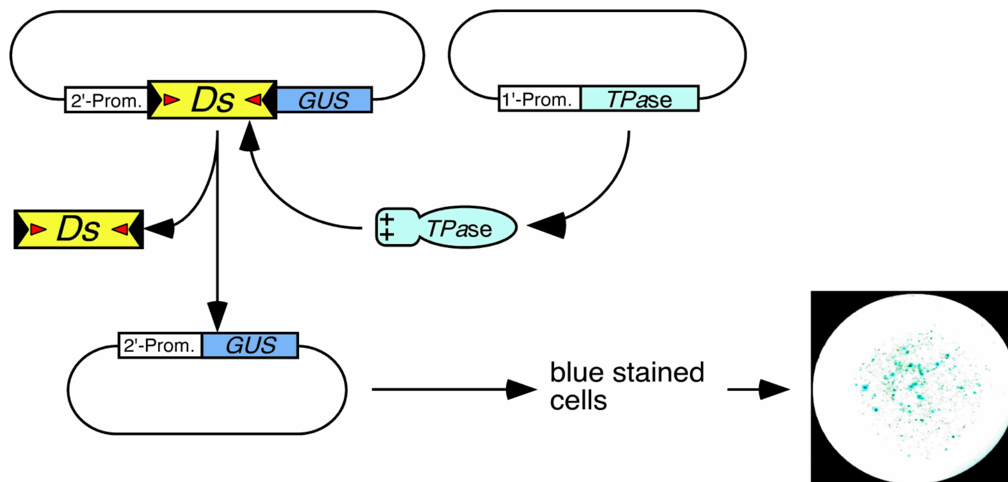


Fig. 6. *Ds* excision assay in petunia protoplasts.

In the *Ds* reporter plasmids, expression of the β-glucuronidase (GUS) gene is blocked by a *Ds* element inserted between the 2'-promoter from *A. tumefaciens* T-DNA (Velten *et al.*, 1984) and the ATG start codon of the gene. The TPase expressing plasmid, pNT600-10.ATG (Kunze *et al.*, 1993), encodes for a truncated version of the TPase protein, $TPase_{103\text{-}807}$ (Li and Starlinger, 1990), lacking the N-terminal 102 amino acids not required for the transposition reaction. This *Ac* TPase derivative was shown to be fully *Ds*-excision-competent and to induce even higher excision rates than the full-length TPase in former petunia transient *Ds* excision assays (Houba-Hérin *et al.*, 1990; Becker *et al.*, 1992). Once the TPase is expressed in the cell, it recognizes *Ds* subterminal regions in the *Ds* reporter plasmids, and activates *in trans* the excision of the element. *Ds* excision is accompanied by reversion to GUS activity that can be detected by a histochemical assay, where a substrate (X-Gluc, 5-Bromo-4-chloro-3-indolyl-beta-D-glucuronic acid), through the enzymatic activity of GUS, is converted to a insoluble, blue dye. After approximately 30 hr culturing time (standard time), aliquots of approximately 4×10^4 of the 10^6 transfected protoplasts

were fixed on nitrocellulose filters and stained for GUS; those expressing GUS were recognized as blue spots on the filter and were counted as unique *Ds* excision events. Normally, two or more filters were spread with protoplasts per transfection. Blue spots appearing on each filter were counted and their average number (among all the filters) was taken as an indication of the transposition frequency of the *Ds* element from the transfected donor reporter plasmid.

2.1.2 Construction of a *Ds* excision reporter vector replicating in petunia cells

As a first step "replicating" and "non-replicating" *Ds* elements were needed in order to investigate in a petunia transfection system the excision competences of methylated or unmethylated *Ds* elements in relation to DNA replication.
Previous studies of *Ds* excision in dicot plants were performed with help of transient transfection assays similar to that employed in this work and with *Ds* excision reporter plasmids that were supposed to be non-replicating in plant cells (Houba-Hérin *et al*., 1990; Houba-Hérin *et al*., 1994a; Houba-Hérin *et al*., 1994b; R. Lütticke and R. Kunze, unpublished).
In this work, *Ds* reporter plasmids resembling those employed in early assays and a new set of *Ds* reporter vectors carrying geminivirus replicon sequences were constructed and scrutinized for their ability to replicate or not in petunia cells.

2.1.2.1 Constructs

<u>pMiDsf1</u>
The plasmid pMiDsf1 (Fig. 7) (8571 bp), derives from the pNT150MiniDs plasmid that was used in former transient petunia assays (Becker *et al*., 1992; Chatterjee and Starlinger, 1995) and carries a uidA (GUS) gene whose expression is blocked by a mini*Ds* transposon that consists of the 246 5'-terminal and the 446 3'-terminal *Ac* residues. The GUS gene expression is under the control of the 2'-promoter. Contiguous to the GUS-gene, the Luciferase gene under the control of the 1'-promoter is inserted. The Luciferase reporter gene has no application in the present work.
In pMiDsf1, the f1 origin of replication of bacteriophage M13 is cloned between the GUS and the Amp resistance genes. This enabled the preparation of single stranded DNA of

pMiDsf1, employed for studies of the excision properties of the *Ds* element in relation to DNA replication and in association with DNA C-methylation (section 2.3).

The f1 origin of replication corresponds to a 414 bp *Ssp* I/*Bgl* I fragment of the phagemid vector pT7T3; the fragment is inserted in the *Ehe* I site at nucleotide 235 of pNT150MiniDs after blunt ending.

The plasmid pMiDs<1f carries the M13 f1 origin of replication in opposite orientation.

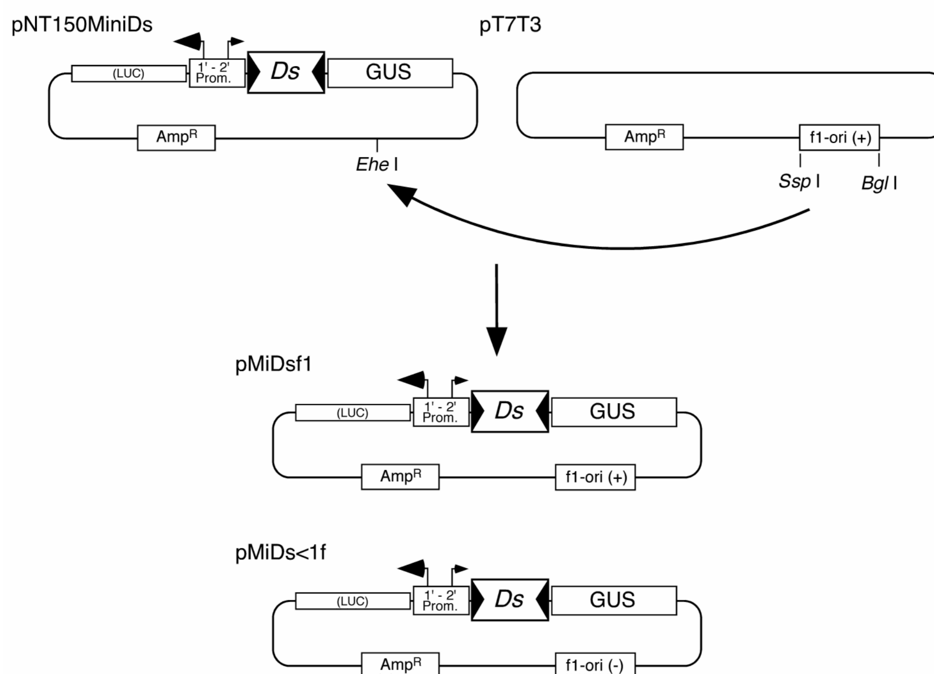


Fig. 7. Construction of pMiDsf1 *Ds* reporter plasmid.

pMiDsf1-RI

The plasmid pMiDsf1-RI (Fig. 9) (8712 bp), derives from the pMiDsf1 plasmid and carries in place of the Luciferase gene the *cis*- and *trans*-acting replication sequences derived from the genome of the monopartite geminivirus Tomato Yellow Leaf Curl Virus (TYLCV) (Kheyr-Pour *et al.*, 1992).

This family of geminiviruses infects diverse dicotyledonous hosts and it is the cause of one of the most devastating virus deseases of cultivated tomatoes (*Lycopersicum esculentum*

Mill.) in the Mediterranean basin, in many subtropical African countries and in Latin America (Czosnek *et al*., 1990).

The structure and coding capacity of its genome is illustrated in Fig. 8. The virus is characterized by a circular single-stranded genome of 2770 bp. In the virion sense orientation (plus-strand) two genes, V1 and V2, are involved in movement and structural functions. In the complementary sense orientation (minus-strand) four *trans*-acting replication open reading frames (ORFs) are found, C1 to C4, that are involved in transcription regulation (in particular C2-C4) and presumably interfere with some cellular processes required for the replicative cycle. The three ORFs C1–C3 partially overlap, and the small ORF C4 is contained in the C1 gene. The sequence C1 encodes for the Replicase protein (Rep) (41 kDa), the only virally encoded protein required for the replication of the geminivirus genome. The two divergent gene clusters are separated by a *cis*-acting intergenic region (IR) of approximately 300 nucleotides that contains the DNA binding site for the Rep protein, the origin of replication, and the eukaryotic promoter signals oriented in both senses (Fontes *et al*., 1992; Thommes *et al*., 1993; Fontes *et al*., 1994).

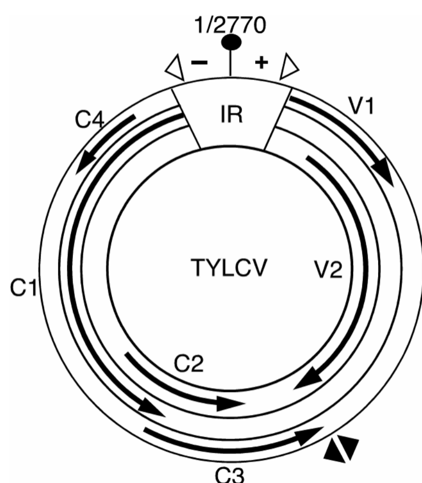


Fig. 8. Genome organisation of TYLCV geminivirus. ORFs are shown as black arrows. The ORFs of plus-strand (virion strand) polarity are designated V1 and V2, ORFs of minus-strand (complementary strand) are designated C2 through C4. IR indicates the intergenic region. Open triangles indicate the positions of eukaryotic promoter signals (CAAT and TATAA boxes) in both (+) and (-) sense orientation. Filled triangles indicate the positions of polyadenylation signals (AATAAA), located at the end of ORFs V2 and C3.

TYLCV DNA replication parallels that of plant chromosomal DNA replication in the following ways: (i) DNA replication of geminiviruses takes place in the cell nucleus (Horns and Jeske, 1991) through a double-stranded intermediate (Laufs *et al*., 1995); (ii) replicating double stranded DNAs of geminiviruses exist as minichromosomes (Saunders *et al*., 1991; Stenger *et al*., 1991; Pilartz and Jeske, 1992), and (iii), except for participation

of the virus-encoded Rep protein that catalyses cleavage and joining at the viral origin of replication (Elmer *et al*., 1988; Laufs *et al*., 1995), geminivirus DNA synthesis relies entirely on the DNA replication apparatus of the host plant (Elmer *et al*., 1988; Etessami *et al*., 1991). Nonetheless, host cell division does not appear to be a prerequisite for geminiviral DNA replication (Nagar *et al*., 1995).

Cloning strategy:

cis- and *trans*-acting replication sequences of the geminivirus were isolated from the clone pTY-Sst14 (Kheyr-Pour *et al*., 1992; EMBL/GenBank accession No. X61153), kindly provided by Bruno Gronenborn (CNRS, Gif sur Yvette, France), and cloned in pMiDsf1 to generate the pMiDsf1-RI construct.

The cloning procedure is illustrated in Fig. 9.

Virion genes V1 and V2 are removed from TYLCV genome by restriction of pTYSst14 with *Nco* I and *Sna* BI. After blunt-ending and dephosphorylation, the remaining plasmid is digested with *Sac* I. Two fragments are generated, of 635 bp and 1279 bp respectively; the first contains the IR *cis*-acting sequences and one half (N-terminal) of the sequence coding for C1, whereas the second carries the C3 ORF and the remaining half (C-terminal) of the C1 coding sequence. By re-ligation of the two fragments at the *Sac* I site, a 1914 bp long ligation product is formed that corresponds to the intact cis and *trans*-acting replication sequences of TYLCV.

This is inserted into the pMiDsf1 plasmid, where the Luciferase gene (1.8 kb) is removed by *Kpn* I/*Ehe* I restriction digestion and the formed plasmid ends blunt ended. The replicon sequences are cloned in opposite orientation to the GUS gene.

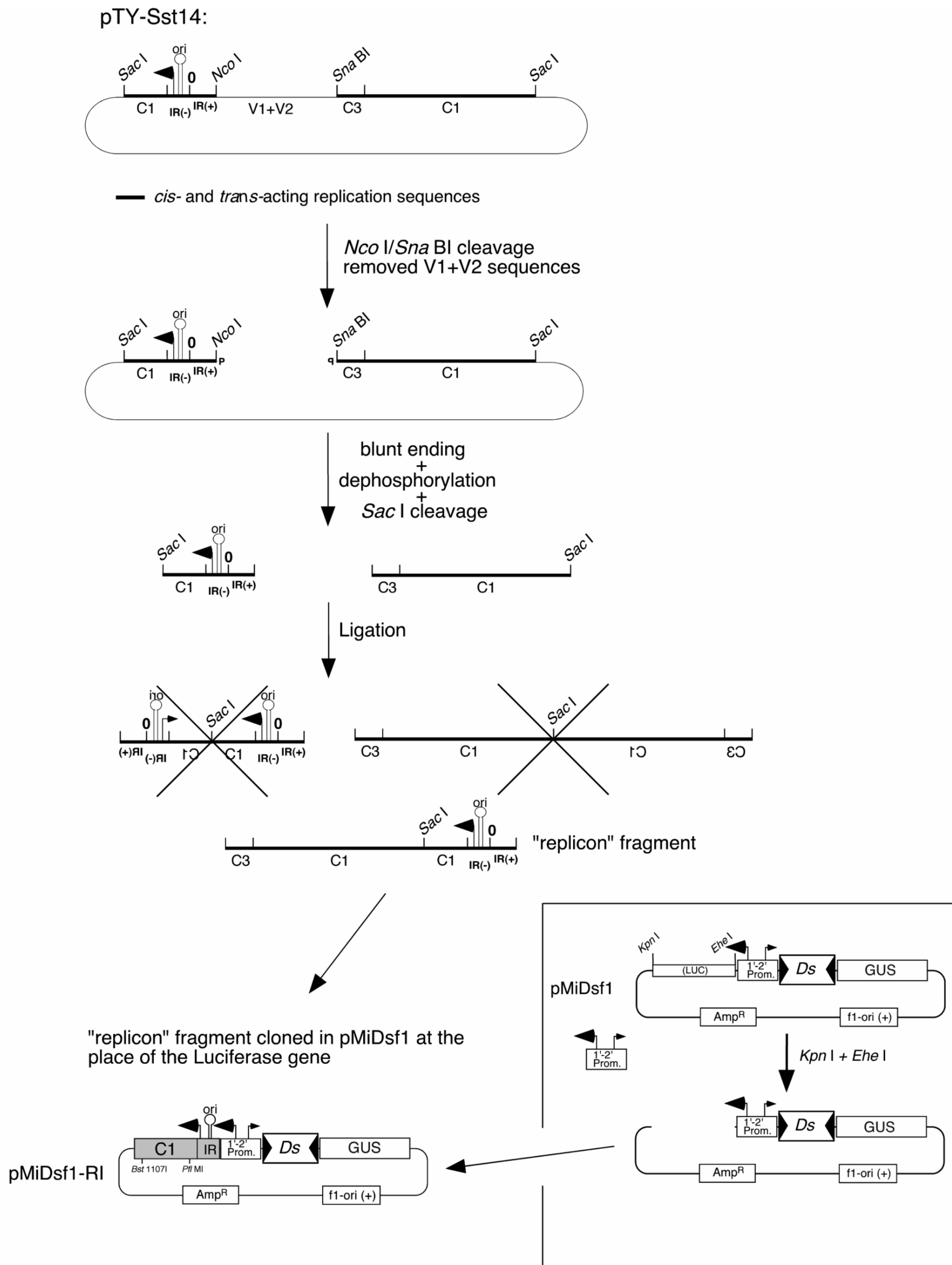


Fig. 9. Cloning strategy of TYLCV replicon sequences in pMiDsf1 to construct pMiDsf1-RI.

Control plasmids

As control, a set of plasmids were prepared: two constructs, pMiDsf1-rI and pMiDsf1-R, derive from pMiDsf1-RI, where the replicon sequences are mutated either at the *trans*-acting region or at the *cis*-acting region respectively.

In pMiDsf1-rI (Fig. 10A) the sequence C1 coding for the Rep protein is inactivated by inverting a 790 bp internal *Bst* 1107I/*Pfl* MI fragment. In pMiDsf1-R (Fig. 10B), the intergenic region is deleted by removal of a *Pfl* MI/*Nco* I 300 bp fragment from pMiDsf1-RI. In a third construct, pMiDsf1-ΔRI, the entire replicon sequence is deleted (Fig. 10C).

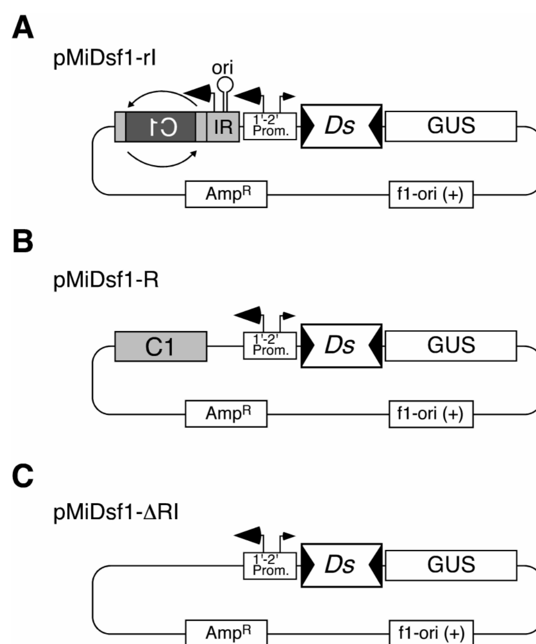


Fig. 10. Control *Ds* reporter plasmids. A) pMiDsf1-rI, **B**) pMiDsf1-R and **C**) pMiDsf1-ΔRI.

2.1.2.2 Analysis of the replication activity of the *Ds* reporter plasmids in petunia cells

The replication activity of pMiDsf1, pMiDsf1-RI, pMiDsf1-ΔRI, pMiDsf1-rI and pMiDsf1-R was tested in petunia protoplasts.

Aliquots of 10^6 petunia protoplasts were transfected with 10 µg *dam-dcm*-methylated *Ds* reporter plasmid DNA, prepared in the *E. coli* strain DH5α. The TPase expression plasmid pNT600-10.ATG, a plasmid not directly involved in this experimental part of the work, was also co-transfected in order to reproduce all the assay conditions applied in the later *Ds* transposition frequency studies. Its DNA was isolated from the *E. coli* strain DH5α.

After transfection, petunia protoplasts were then scrutinized for the appearance of replicated *Ds*-reporter plasmids. The procedure was the following: the restriction enzyme *Mbo* I only digests DNA containing unmethylated adenine residues within its recognition sequence, GATC. Therefore, transfected plasmid DNA is initially resistant to *Mbo* I digestion, but after a few replication cycles in petunia cells, the plasmid become sensitive to *Mbo* I digestion due to the loss of *dam*-methylation.

Total DNA isolated from the whole 10^6 transfected protoplasts was digested with *Mbo* I enzyme and subjected to Southern Blot hybridization, using a 1024 bp *Mbo* I fragment of the *Ds* reporter plasmids as probe. The *Mbo* I restriction fragment employed is shown in Fig. 11A. It completely hybridizes with all *Ds* reporter plasmids transfected and only partially with the TPase expression vector. That is why a signal corresponding to the undigested pNT600-10.ATG co-transfected plasmid is always observed after Southern detection.

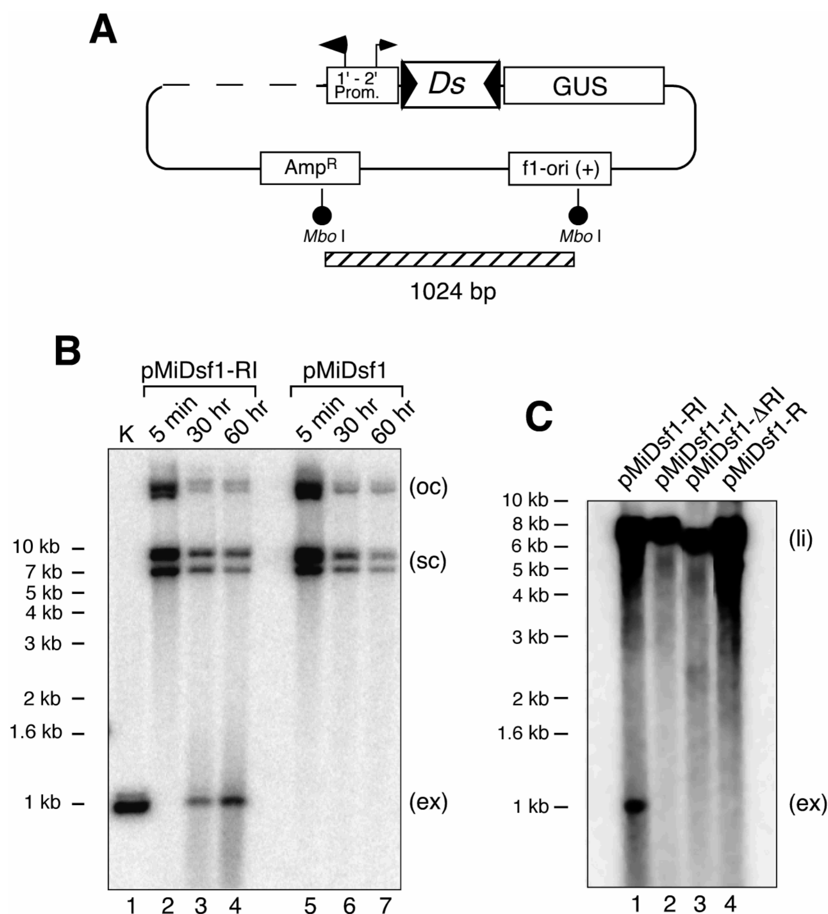


Fig. 11. The TYLCV Rep protein and replication origin are active in petunia protoplasts. A) The 1024 bp *Mbo* I restriction fragment of *Ds* reporter plasmids employed for Southern detection of plasmid replicative forms. It partially hybridizes also with the TPase expression plasmid pNT600-10.ATG. **B)**

DNA isolated from petunia protoplasts at different timepoints after cotransfection with the TPase expression plasmid pNT600-10.ATG and pMiDsf1-RI (lanes 2-4) or pMiDsf1 (lanes 5-7) was digested with the *dam*-methylation sensitive enzyme *Mbo* I, separated by electrophoresis on a 1% agarose gel, blotted and hybridized with the probe shown in (A), that hybridizes with the same *Mbo* I fragment in all reporter plasmids. Lane 1: unlabelled probe fragment (K). oc: undigested, open circle forms of TPase expression plasmid (7.8 kb) and *Ds* reporter plasmid; sc: undigested, supercoiled TPase expression plasmid and *Ds* reporter plasmid; ex: 1 kb *Mbo* I digestion product of the reporter plasmids. **C)** Gel blot analysis of DNA isolated from petunia protoplasts 30 hr after transfection with pMiDsf1-RI (lane 1) or the replication-deficient mutants pMiDsf1-rI (lane 2), pMiDsf1-ΔRI (lane 3) and pMiDsf1-R (lane 4) respectively. The DNA was digested with *Mbo* I and *Sca* I, separated by electrophoresis, blotted and hybridized with the probe shown in (A). li: linearized TPase expression and *Ds* reporter plasmids.

Fig. 11B shows the digestion products of pMiDsf1 and pMiDsf1-RI 5 min, 30 hr and 60 hr after transfection. The re-isolated pMiDsf1 DNA is resistant to *Mbo* I digestion at each time after transfection, indicating that the plasmid does not replicate in plant cells (lane 5-7).

In contrast, *Mbo* I releases increasing amounts of the 1024 bp digestion fragment from pMiDsf1-RI DNA isolated 30 and 60 hr after transfection, indicating that the plasmid in this case replicates in plant cells (lanes 2-4). However, completely undigested plasmid DNA is still detected. This means that either not all the transfected plasmid molecules are replicating once (*Mbo* I is not sensitive to hemimethylated restriction sites); or residual untransfected plasmid molecules are contaminating the protoplast DNA extractions; or, both effects are involved.

To ensure that the replication activity of pMiDsf1-RI is really dependent on the functionality of the viral sequences inserted in the plasmid, the three plasmids, pMiDsf1-rI, pMiDsf1-R and pMiDsf1-ΔRI, defective respectively in the *cis*-acting, *trans*-acting or both TYLCV replication sequences, were tested. In this case the DNA analyses were performed 30 hr after transfection and in addition to *Mbo* I a second *dam*-methylation insensitive enzyme, *Sca* I, that linearizes the *Mbo* I undigested TPase expression plasmid and *Ds* reporter plasmids was employed. The result in Fig. 11C confirms the expectations, since no 1024 bp signal is detectable in correspondence of the three control plasmids.

These data show that TYLCV Rep protein and the origin of replication function in petunia cells. They enabled the creation of a *Ds* excision plasmid, pMiDsf1-RI, replicating in protoplasts. Without these sequences, no replication occurs. This indicates that plasmids used in former petunia transfections were non-replicating (Houba-Hérin *et al.*, 1990; Becker *et al.*, 1992; Kunze *et al.*, 1993; Heinlein *et al.*, 1994; Chatterjee and Starlinger, 1995).

2.2 Effects of DNA replication on *Ac/Ds* transposition in the absence of transposon C-methylation

2.2.1 DNA replication improves *Ds* transposition in a host-specific way

Transient transfection assays performed in monocot and dicot systems showed apparently conflicting results about the dependence of *Ac/Ds* transposition on DNA replication in the two host species (Houba-Hérin *et al*., 1990; Laufs *et al*., 1990; Becker *et al*., 1992; Kunze *et al*., 1993; Heinlein *et al*., 1994; Houba-Hérin *et al*., 1994a; Houba-Hérin *et al*., 1994b; Chatterjee and Starlinger, 1995; McElroy *et al*., 1997; Wirtz *et al*., 1997; R. Lütticke and R. Kunze, unpublished).

To understand the behaviour of *Ds* transposon in dicots in a 'replication' and 'non-replication' context, petunia protoplast transient assays were performed with pMiDsf1 and pMiDsf1-RI and control plasmids. In this way it was possible to verify whether also in dicots replication plays a role in regulating the competence of *Ac/Ds* elements to transpose, and this independently from the methylation status of the element. The frequency with which the *Ds* element could excise from each donor plasmid was studied using the GUS-staining assay and DNA analysis.

2.2.1.1 *Ds* excision activity in pMiDsf1, pMiDsf1-RI and control plasmids detected by GUS-staining

Several independent petunia assays were performed with the replicating and non-replicating reporter plasmids. In every single assay, aliquots of 10^6 petunia protoplasts were co-transfected with 10 µg of the TPase expression plasmid pNT600-10.ATG and respectively 10 µg of each *Ds* excision reporter plasmid. *Ds* reporter plasmid DNA used for transfection was prepared in the *Dam⁻-Dcm⁻ E. coli* strain JM110. This was to ensure an end result independent of any DNA methylation.

Fig. 12 shows the outcome of one of these assays, where *Ds* excisions from pMiDsf1 and pMiDsf1-RI plasmids were compared at different times after transfection.

Tab. 2 shows the results of the GUS-staining of different assays carried out 30 hr after transfection.

In the experiment depicted in Fig. 12, protoplasts were spread on the nitrocellulose 5 min, 30 and 60 hr after transfection. They were then stained and inspected for frequency and

staining intensity of GUS-positive (blue) cells. The values shown below each filter represent the number of blue protoplasts counted at each collection time and for pMiDsf1 or pMiDsf1-RI. No *Ds* excision is detectable 5 minutes after transfection. Thirty hours after transfection, *Ds* excision is observed in both plasmids, and between 30 and 60 hr this number does not significantly increase any more. However the frequency of blue staining cells with the replicating plasmid pMiDsf1-RI is approximately 2 to 3.5 times higher compared to the non-replicating plasmid (Fig. 12, Tab. 2). Moreover, the staining intensity of the protoplasts is significantly higher when transfected with the replicating plasmid (Fig. 12).

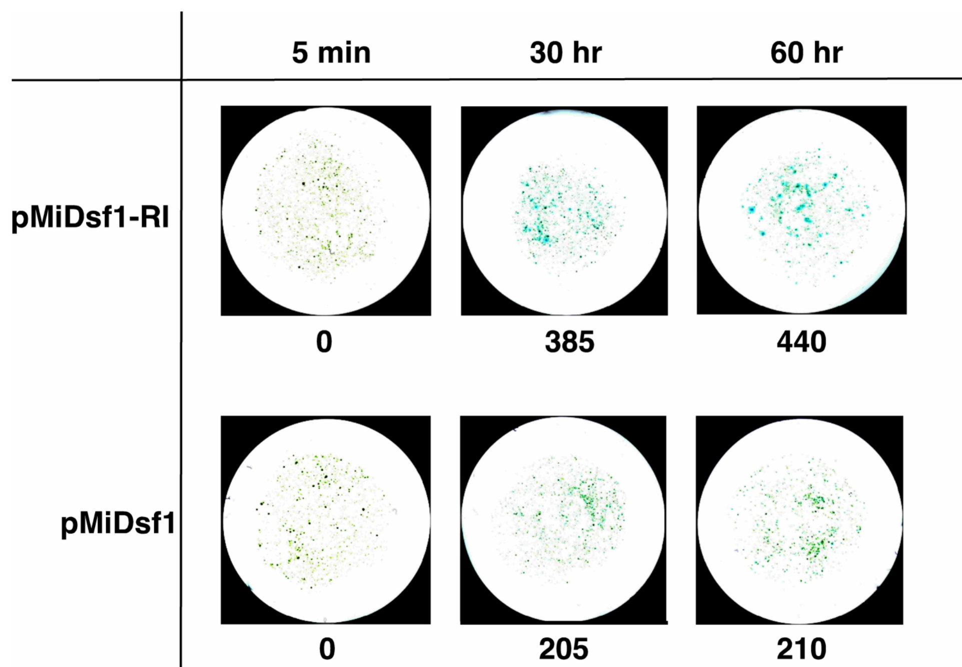


Fig. 12. GUS-assay of protoplasts transfected with the replicating pMiDsf1-RI and the non-replicating pMiDsf1 reporter plasmid. Petunia protoplasts were co-transfected with 10 µg pNT600-10.ATG and 10 µg pMiDsf1 or pMiDsf1-RI respectively. Five minutes, 30 and 60 hr after transfection, protoplasts were spread on two nitrocellulose filters and GUS-stained. One of the two filters is shown for each reporter construct and collection time and the values indicated below each filter represent the average number of total blue-stained protoplasts counted on the two filters.

The frequencies of *Ds* transposition for the two non-replicating plasmids pMiDsf1-rI and pMiDsf1-R are always comparable with that observed for pMiDsf1 (Tab. 2, Exp. I and II). Unespectedly, *Ds* excision from the non-replicating vector pMiDsf1-ΔRI is about 4-fold

reduced. It is conceivable that the modified sequence environment around the transposon is responsible for this reduction ("position effect").

Tab. 2. GUS-positive protoplasts after transfection with the replicating pMiDsf1-RI and the non-replicating reporter plasmids. Petunia protoplasts were co-transfected with 10 µg pNT600-10.ATG and 10 µg pMiDsf1 or pMiDsf1-RI, pMiDsf1-rI, pMiDsf1-R and pMiDsf1-ΔRI respectively. Thirty hours after transfection, protoplasts were spread on nitrocellulose filters and GUS-stained. Each value represents the average number of protoplasts counted on two filters. The results of six independent experiments are shown.

Ds excision events of replicating and non-replicating *Ds* elements in petunia protoplasts						
	number of GUS-positive cells					
	Exp. I	Exp. II	Exp. III	Exp. IV	Exp. V	Exp. VI
pMiDsf1	53	42	130	127	135	165
pMiDsf1-RI	135	140	319	323	275	435
pMiDsf1-rI	52.5	60				
pMiDsf1-R	48.5	40				
pMiDsf1-ΔRI	13	10				

These results reveal several aspects of the experimental system: first of all they finally prove that in petunia, a dicot species, the *Ds* element can actively transpose also from plasmids that do not replicate in plant cells. However, also in petunia replication stimulates *Ds* excision, as directly indicated by the 2- to 3.5-fold higher number of blue protoplasts observed with pMiDsf1-RI in comparison to pMiDsf1. The higher blue staining intensity of the protoplasts transfected with the replicating construct seems to support this notion.

However, GUS-staining can not distinguish if the boost in GUS-positive cells observed in the presence of replication reflects an increased *Ds* excision frequency, or is a consequence of a replicative amplification of plasmids before *Ds* excision (that increases the probability that one *Ds* excision event occurs per cell) or after *Ds* excision (that renders protoplasts with unique *Ds* excision event recognizable, which would otherwise be undetectable). To distinguish between these possibilities a quantitative DNA analysis has to be performed.

2.2.1.2 *Ds* excision activity in pMiDsf1 and pMiDsf1-RI detected by DNA analysis

Protoplasts were co-transfected with pNT600-10.ATG together with pMiDsf1 or pMiDsf1-RI. Thirty hours after transfection they were treated with DNase I to destroy residual plasmid DNA in the medium and total DNA was extracted. This DNA contained, in

addition to genomic DNA and TPase expression vector, a mixture of *Ds* reporter plasmids where *Ds* either did or did not excise.

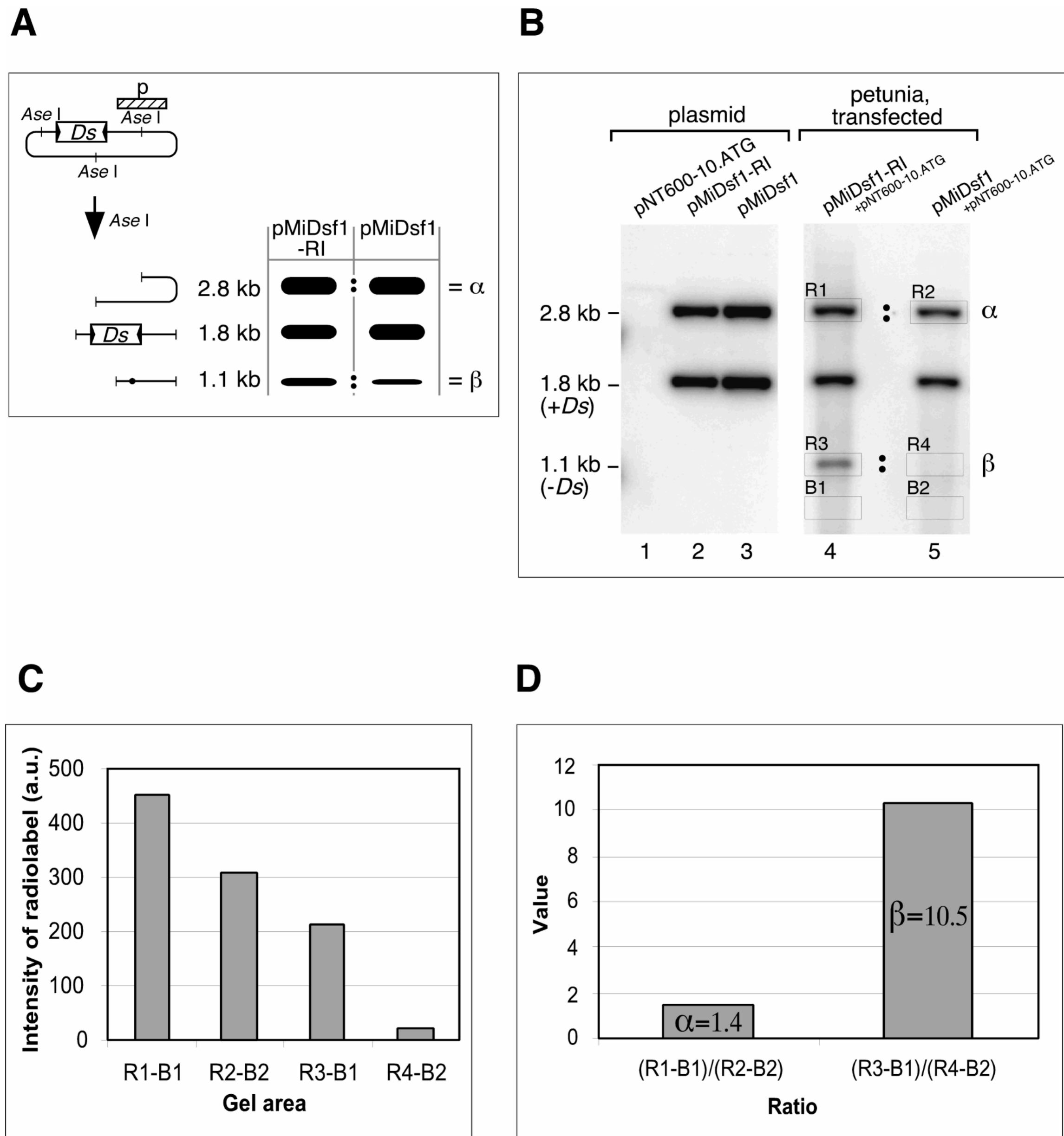


Fig. 13. Replication enhances *Ds* excision. A) Schema of the quantitative determination of *Ds* excision from pMiDsf1 and pMiDsf1-RI. Petunia protoplasts were co-transfected with pNT600-10.ATG and pMiDsf1 or pMiDsf1-RI respectively. Total protoplast DNA was isolated and digested with *Ase* I and analysed by gel blot hybridization 30 hr after transfection. The probe (*p*) hybridizes with all three reporter plasmid digestion products; a schematic of the gel picture is shown to the right of the digestion products. α, ratio of plasmid DNA extracted from the cells; β, ratio of empty donor site fragments. **B)** Gel blots of *Ase* I digested pNT600-10.ATG, pMiDsf1-RI and pMiDsf1 plasmid DNA as controls (lanes 1 to 3), and DNA from petunia protoplasts cotransfected with pNT600-10.ATG and pMiDsf1-RI or pMiDsf1 (lanes 4

and 5). α: ratio of 2.8 kb vector bands in lanes 4 and 5; β: ratio of 1.1 kb *Ds* excision bands in lanes 4 and 5. The intensity of radiolabel in gel areas R1, R2, R3, R4, B1 and B2 was determined. **C)** Quantification of the intensity of radiolabel (arbitrary units, a.u.) in gel areas R1, R2, R3 and R4. B1 and B2 were used as background and subtracted from band signals R1/R3 and R2/R4, respectively. **D)** According to signal intensities, ratios $\alpha=(R1-B1)/(R2-B2)=1.4$ and $\beta=(R3-B1)/(R4-B2)=10.5$ were calculated. Finally, replication improves *Ds* transposition by at least a factor of 7.5 (β/α).

After digestion with *Ase* I restriction enzyme and Southern blotting, DNA was hybridized with a probe that detects three fragments (Fig. 13A and B): a 2807 bp vector fragment whose abundance reflects the total amount of plasmid, a 1798 bp fragment from plasmids that still contain the *Ds*, and a 1089 bp fragment from plasmids where *Ds* has been excised (Fig. 13A). To achieve comparable hybridization signals, the probe was designed to cover segments of the 2.8 kb and 1.1 kb fragments that are nearly identical in length and G/C-content. It corresponds to a 647 bp long, *Msc* I/*Ssp* I restriction fragment of the *Ds* reporter plasmid and does not hybridize with the co-transfected TPase expressing plasmid (Fig. 13B, lane 1). The ratio α of the 2807 bp-band-intensities between pMiDsf1- and pMiDsf1-RI-transfected cells reflects the overall difference in plasmid content of the samples due to replication (copy number effect) and aliquot size. The ratio of the 1089 bp-band-intensities (β) corresponds to the product of copy number effect, change in excision frequency and aliquot size. The ratio β/α indicates the factor by which the *Ds* excision frequency is promoted (or deprived) by replication of the reporter plasmid pMiDsf1-RI. After Southern blotting, the respective bands were quantified. In Fig. 13B, the areas used for the quantification procedure are indicated with a frame. Fig. 13C shows the signal intensity in gel areas R1, R2, R3 and R4 from which the respective background was subtracted. Ratios $\alpha=1.4$ and $\beta=10.5$ are the outcome of (R1-B1)/(R2-B2) and (R3-B1)/(R4-B2), respectively (Fig. 13D).

These data indicate that *Ds* excision frequency in petunia cells is promoted by at least 7.5-fold by replication of the host plasmid. This value is presumably an underestimate because the 1089 bp *Ds* excision band in lane 5 was not detectable by Southern blotting and thus the excision band in lane 4 was divided by a background signal.

To investigate whether this replication-mediated transposition boost is independent of the methylation state of the transfected plasmids and/or *Ds* DNAs, a restriction and Southern analysis was carried out on total DNA of the transfected protoplasts as described in Fig. 14A. Total DNA was extracted from protoplasts 30 hr after transfection with pMiDsf1 and

pMiDsf1-RI. The DNA was then digested with the C-methylation sensitive restriction enzyme *Bsi* EI and subjected to Southern blot analysis. In the absence of C-methylation, this enzyme cuts pMiDsf1 and pMiDsf1-RI 6 times in the *Ds* element and 8 times in the vector.

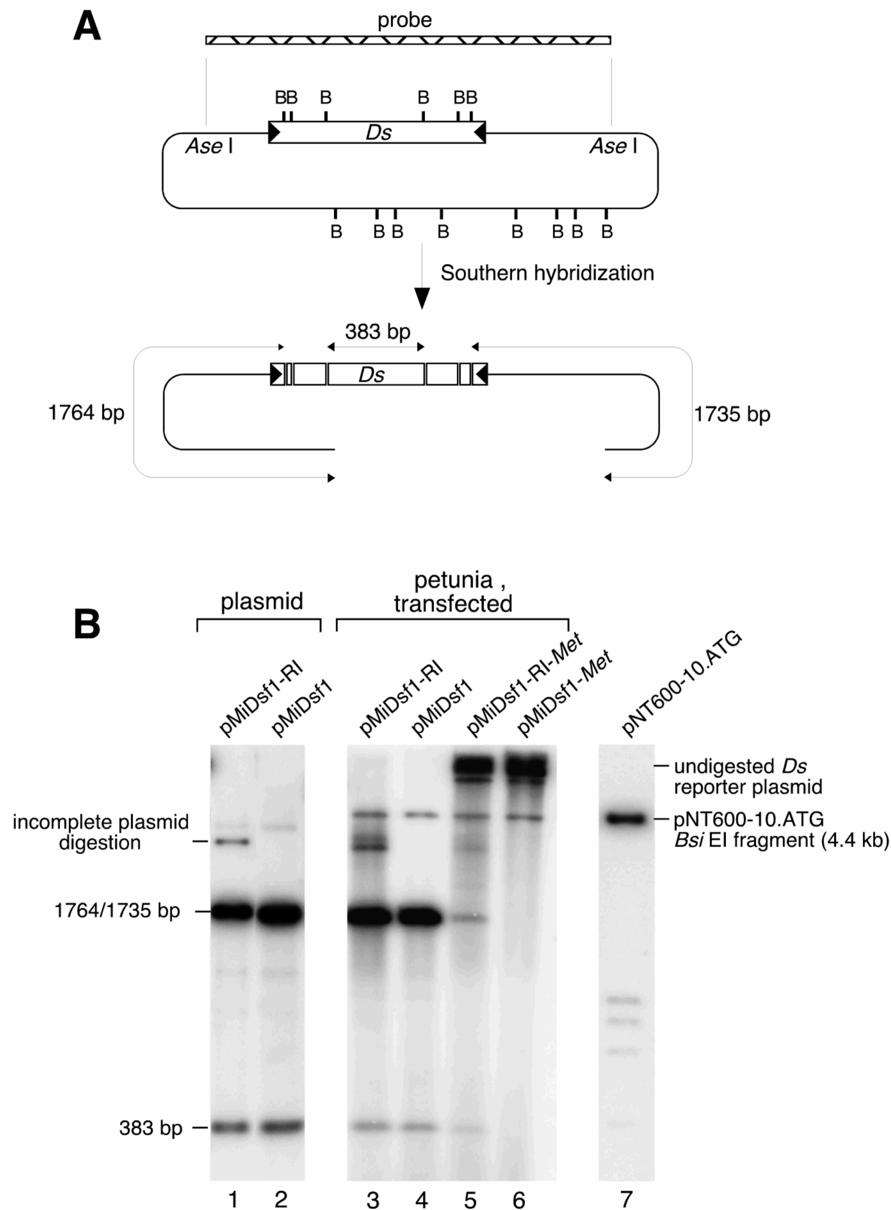


Fig. 14. Replication-mediated transposition boost is independent of the methylation state of the transfected plasmids. A) Schematic of the *Bsi* EI digestion assay to test the C-methylation status of the reporter plasmids after transfection in plant protoplasts. In the absence of C-methylation, *Bsi* EI (B) cuts pMiDsf1 and pMiDsf1-RI 6 times in the *Ds* element and 8 times in the rest of the vector. The probe for Southern hybridization is a 1798 bp long *Ase* I restriction fragment of the plasmids which detects either *Ds* digestion products and the entire undigested plasmid. The detected *Ds* digestion products correspond

to the three longer fragments of 383, 1735 and 1764 bp, respectively. **B)** Southern Blot hybridization of *Bsi* EI restricted DNA extracted from protoplasts co-transfected with pNT600-10.ATG and C-unmethylated pMiDsf1-RI (lane 3) and pMiDsf1 (lane 4) or CpG methylated pMiDsf1-RI-*Met* (lane 5) and pMiDsf1-*Met* (lane 6) plasmids respectively; lane 1 and 2, unmethylated pMiDsf1-RI and pMiDsf1 plasmids respectively, digested with *Bsi* EI enzyme before transfection; lane 7, pNT600-10.ATG TPase expression vector digested with *Bsi* EI before transfection.

The probe choosen for Southern hybridization corresponds to a 1798 bp long *Ase* I restriction fragment of the plasmids that detects the *Ds Bsi* EI digestion products and the undigested plasmid. In addition, it hybridizes to a 4.4 kb *Bsi* EI digestion product of pNT600-10.ATG TPase expression plasmid (Fig. 14B lane 7) that was co-transfected with the *Ds* reporter plasmids (lane 3, 4, 5 and 6).
As indicated in Fig. 14B, pMiDsf1-RI and pMiDsf1, C-unmethylated before transfection (lanes 1 and 2), remain unmethylated (lane 3 and 4) in the host cells 30 hr after transfection: the same *Ds Bsi* EI plasmid digestion products appear before and after transfection. They correspond to the three detectable fragments of 1764 bp, 1735 bp (indistinguishable on the gel) and 383 bp. No signal of entire undigested plasmid is visualized anymore by Southern blotting. In lane 3, two weak additional bands longer than 1.8 kb are observed which are most likely products of incomplete digestion. Since they appear also in control lane 1, loaded with the unmethylated untransfected plasmid, they are independent of any host *de novo* methylation activity. As a control, lane 5 and 6 were loaded with *Bsi* EI-digested total DNA of protoplasts transfected with C-methylated plasmids. C-methylation was performed *in vitro* with use of *Sss* I methylase (section 2.3.1.1). As expected, only the entire undigested plasmid is detected in the case of pMiDsf1-*Met* (lane 6), thus demonstrating that methylation provided *in vitro* at cytosine residues is stably maintained in plant cells in the absence of replication. In contrast pMiDsf1-RI-*Met* is partially demethylated as a consequence of its replication activity (lane 5). As is in more detail illustrated in section 2.3.1.2, C-methylation does not completely prevent the Rep protein from being expressed and activating plasmid replication. A major fraction of the plasmid remains undigested, however.
These data prove that C-methylation does not account for the *Ds* transposition boost observed during replication.

The GUS-assay always indicated a 2- to 3.5-fold increase of stained protoplasts in the presence of replication, whereas Southern blotting indicates a 7.5-fold increase in *Ds*

excision frequency. The result of the GUS-assays represents an underestimation of the real increase of *Ds* excision frequency: each blue protoplast counts as a single excision event. Actually what the GUS-assay detects is the number of cells containing at least one *Ds* excision; it does not detect the number of independent *Ds* excision events per cell and their eventual amplification by replication. Southern quantification is not subject to these limitations.

To summarise, these studies prove that in petunia cells, a dicot species, independently of the C-methylation status of the transposon DNA, *Ds* excision is promoted by replication. However, the element can actively transpose also in the absence of replication. Since in monocot transient assays, replication was shown to be the prerequisite for *Ac*/*Ds* activity, we can finally conclude that replication influences *Ac*/*Ds* transposition and this occurs in a host-specific manner.

2.2.2 DNA replication influences the formation of transposon footprints

Ac/*Ds* transposition is positively influenced by replication; an increased transposition frequency of the element is the result of some unknown molecular effects of replication on the transposition mechanism. One determinant step of transposition consists of the excision of the element. By excision, *Ac*/*Ds* elements leave characteristic footprints at the donor site. Analysis of the footprints left by the element at the donor site in maize and transgenic Arabidopsis plants has been very useful to investigate the mechanism with which the transposon excises. In this work it has been employed to investigate whether replication has an influence on this mechanism. Footprints formed by *Ds* in pMiDsf1 and pMiDsf1-RI plasmids were analysed.

Petunia protoplasts were co-transfected with TPase expression plasmid and pMiDsf1 or pMiDsf1-RI. Genomic protoplast DNA was isolated 30 hr after transfection. Using standard conditions, the *Ds* excision products from the non-replicating plasmid was not detectable by gel blot hybridization (Fig. 13) or by PCR (Fig. 15B). Therefore optimized reaction conditions were developed that allowed the selective amplification of rare excision site sequences among a large background of wild type plasmids. 150 ng of genomic protoplast DNA were used as template for a PCR reaction where the two primers hybridize to the 2'-promoter and to the GUS gene of the transfected *Ds* reporter plasmids

(Fig. 15A). The two DNA products they could co-amplify consist of the empty donor site (300 bp) and the donor site with *Ds* still inserted (1000 bp). The denaturation step at 95°C for 30 seconds, was followed by a combined annealing and extension step at 60°C for 20 seconds. The temperature cycle was performed 25 times. These conditions (no extension step at 72°C) allowed the selective amplification of the *Ds* empty donor site of 300 nucleotides (Fig. 15C).

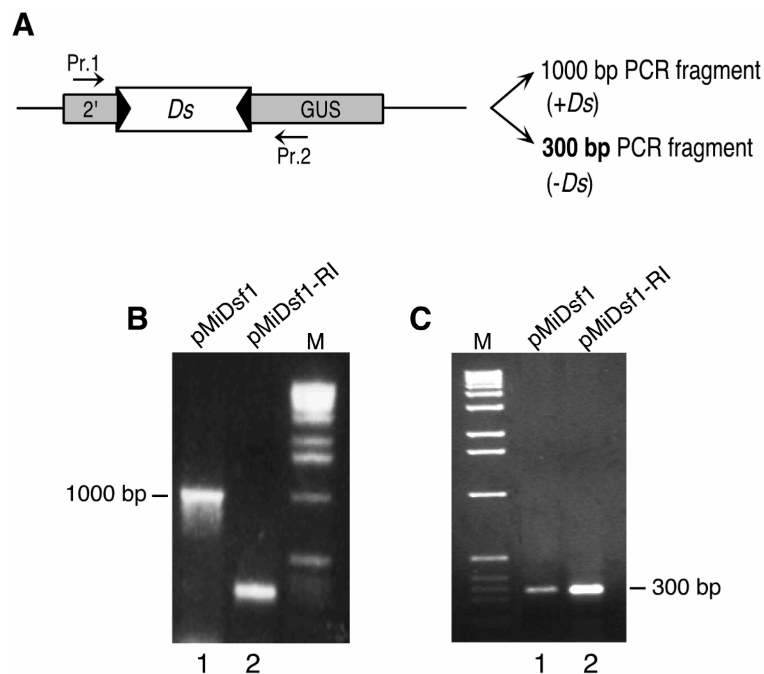


Fig. 15. Establishment of selective PCR conditions to amplify the *Ds* empty donor site. A) To study footprints left by a replicating and a non-replicating *Ds* element on the donor site, a PCR reaction was performed on total DNA extracted from protoplasts 30 hr after transfection with pMiDsf1 and pMiDsf1-RI. By using two primers (Pr.1 and Pr.2), annealing respectively to the 2'-promoter and to the GUS gene of the transfected *Ds* reporter plasmids, both the empty donor site (300 bp) and the donor site with *Ds* still inserted (1000 bp) could be amplified. **B)** Ethidium bromide staining of the *Ds* empty donor site amplified under normal PCR conditions. Under normal PCR conditions (denaturation step at 95°C for 30 seconds, followed by a annealing step at 60°C for 30 seconds and an extension step at 72°C for 30 seconds) only (lane 2) the empty donor sites from the replicating plasmid can be amplified. From the non-replicating plasmid (lane 1) just the 1000 bp product is readily amplified. **C)** Ethidium bromide staining of the *Ds* empty donor site amplified under selective PCR conditions. Selective PCR conditions (a unique annealing and extension step performed at 60°C for 20 seconds) were employed in order to selectively amplify the rare excision site sequences among the huge majority of wild type plasmids. In this way, also from pMiDsf1 non-replicating plasmid (lane 1) the 300 nucleotides *Ds* excision product is detectable and the donor site, with *Ds* still inserted, is extremely underrepresented.

Individual amplified molecules were ligated into pCR2.1-Topo (Topo TA cloning vector, Invitrogen) and transformed into *E. coli*. Random clones were tested for the insertion of the 300 bp excision fragment and footprints were sequenced (Fig. 16).

A

pMiDsf1-RI

	GTACGTGGGGCGCGTTGCGTGACC	5' CAG. . *Ds*. .CTA 3'	GCGTGACCCGGCCGCGCGGATCCGTCGACTCT
55	GTACGTGGGGCGCGTTGCGTGAC	gc	CGTGACCCGGCCGCGCGGATCCGTCGACTCT
5	GTACGTGGGGCGCGTTGCGTGAC	g	GCGTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGTGGGGCGCGTTGCGTG	c	CGTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGTGGGGCGCGTT	ttgatatgagtctggaaggagagtttacag	TCT
1	GTACGTGGGGCG		TCGACTCT
1	GTACGTGGGG		CGTCGACTCT
1	GTACGTGGGGCGCG	aac	CGTGACCCGGCCGCGCGGATCCGTCGACTCT

B

pMiDsf1

	GTACGTGGGGCGCGTTGCGTGACC	5' CAG. . *Ds*. .CTA 3'	GCGTGACCCGGCCGCGCGGATCCGTCGACTCT
33	GTACGTGGGGCGCGTTGCGTGAC	gc	CGTGACCCGGCCGCGCGGATCCGTCGACTCT
10	GTACGTGGGGCGCGTTGCGTGAC	g	GCGTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGTGGGGCGCGTTGCGTGAC	g	GTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGTGGGGCGCGTTGCGTGAC	ggc	CGTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGTGGGGCGCGTTGCGTGAC		GACCCGGCCGCGCGGATCCGTCGACTCT
2	GTACGTGGGGCGCGTTGCGTGAC		CGTGACCCGGCCGCGCGGATCCGTCGACTCT
3	GTACGTGGGGCGCGTTGCGTGAC		GTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGTGGGGCGCGTTGCGTGAC	gaaccgagtcctatttactgaacccagtgggatca	CGTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGTGGGGCGCGTTGCGTGA	acg	GTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGTGGGGCGCGTTGCGTG	c	CGTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGTGGGGCGCGTTGCG	agaccc	CGTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGTGGGGCGCGTTGCG		CGTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGTGGGGCGCGTTG		CGTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGTGGGGCGCGTTG		CCCGGCCGCGCGGATCCGTCGACTCT
2	GTACGTGGGGCGCGTT		GCGTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGTGGGGCGCGTT		GTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGTGGGGCGCG	c	CGTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGTGGGGCGC	aac	CGTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTACGT		CGTGACCCGGCCGCGCGGATCCGTCGACTCT
1	GTAC		CGTGACCCGGCCGCGCGGATCCGTCGACTCT

Fig. 16. Transposon footprints left after excision by a replicating and non-replicating *Ds* element. **A)** Footprints from the replicating pMiDsf1-RI plasmid. **B)** Footprints from the non-replicating pMiDsf1

plasmid. The top lines show the sequence at the *Ds* insertion site. The 8 bp TSDs are highlighted at both *Ds* borders. Lines below show empty donor site sequences of independent *Ds* excision events. The numbers to the left indicate the frequency of each excision footprint.

This analysis also allowed the determination of the relative abundance of particular template molecules in the pool of amplified molecules. Total DNA from 4 independent petunia transfection assays was extracted and on each DNA preparation one to two polymerase chain reactions were done. Once each amplification product was ligated into pCR2.1-TOPO and the ligation product was transformed in *E. coli*, the plasmid inserts from 7 to 18 colonies were sequenced per transformation. Thus for each plasmid, 65 independent footprints were analysed in total.

During replication, one predominant footprint ('*gc*') is formed in 84% of excision events, 8% contain a second type ('*g*'), and 8% of footprints have individually different sequences (Fig. 16A). The '*gc*' and '*g*' footprints dominate with frequencies of 51% and 15% also in the absence of replication; however, the frequency of deviating and individually different footprints, showing deletions varying in size as also diverse sequence insertions, is almost 5-fold higher (34%) (Fig. 16B) than in the presence of replication. Moreover, deletions appear to involve predominantely the left border (5') of the *Ds* insertion point, whereas, in the presence of replication no significant bias between the left (5') and the right (3') borders of the insertion point is apparent.

These data suggest that replication has an influence on excision site repair. The efficiency and fidelity of the reactions leading to the formation of the predominant footprints are enhanced.

2.3 Effects of DNA replication on the transposition of methylated *Ac*/*Ds* elements

Genetic experiments with *Ac* in maize have led to the conclusion that *Ac* transposes during or immediately after replication, and that only one of the two resulting sister elements is transposition competent (Greenblatt and Brink, 1963; Greenblatt, 1984; Chen *et al.*, 1987; Chen *et al.*, 1992).

Molecular studies led to the hypothesis that the DNA methylation status of *Ac*/*Ds* elements determines their transpositional competence before and after replication, at least in maize,

and a model was presented (Wang *et al*., 1996; Wang and Kunze, 1998). However, the studies of *Ac*/*Ds* transposition from geminivirus vectors in monocots and the present work suggest that transposition is regulated by replication also in the absence of methylated cytosines.
To understand what role DNA methylation plays for *Ac*/*Ds* events, the effects of DNA methylation on *Ds* transposition were tested *in vivo* in the presence and absence of replication.

2.3.1 CpG methylation of the *Ds* element inhibits transposition

According to the proposed model (Wang *et al*., 1996; Wang and Kunze, 1998), C-methylation of TPase binding sites on both DNA strands should inhibit transposition in the absence of replication; this is the prerequisite for the validity of the model. To challenge this assertion, non-replicating holo-methylated *Ds* elements were prepared and tested for their capability to excise in a petunia protoplast assay. As a control, replicating holo-methylated *Ds* were also produced.

2.3.1.1 Preparation of holo-methylated *Ds* elements

pMiDsf1 and pMiDsf1-RI plasmids were treated *in vitro* with *Sss* I-Methylase that methylates all cytosine residues to ^{5m}C within the double-stranded dinucleotide recognition sequence 5'...CG...3' in the presence of S-adenosylmethionine (SAM). In *Ac*/*Ds* elements almost all CpG motifs are located in the *cis*-acting terminal regions that include the subterminal TPase binding sites (Kunze *et al*., 1988). After the reaction all CpG dinucleotides in the plasmids, including the *Ds* element, are methylated. The reaction was performed at 37°C for 16 hours and every four hours it was supplemented with fresh SAM. As a control, mock-methylated DNA of the two *Ds* reporter plasmids was prepared under the same conditions used in the methylation reaction, except for the presence of *Sss* I-Methylase in the reaction mixture.
In this way pMiDsf1-*Met*, pMiDsf1-RI-*Met*, pMiDsf1-mock and pMiDsf1-RI-mock *Ds* reporter plasmids were created. Methylation and mock-methylation were confirmed by digestion of the reaction products with CpG-methylation-sensitive restriction enzymes *Hpa* II (Fig. 17).

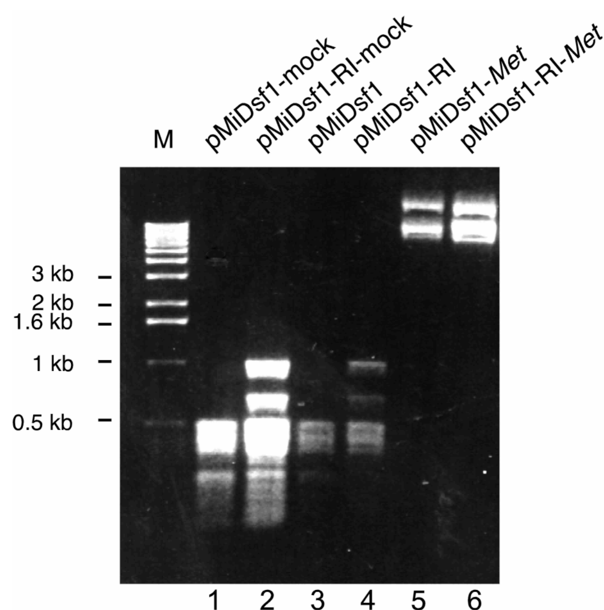


Fig. 17. Ethidium bromide staining of *Hpa* II digestion of *Sss* I-methylated and mock-methylated *Ds* reporter plasmids. Before transfection into petunia protoplasts, methylation and mock-methylation of pMiDsf1-*Met*, pMiDsf1-RI-*Met*, pMiDsf1-mock and pMiDsf1-RI-mock *Ds* reporter plasmids was tested by *Hpa* II restriction digestion. *Hpa* II enzyme recognizes 42 and 37 restriction sites in pMiDsf1 and pMiDsf1-RI respectively. Fragments are generated ranging between 17 and 500 bp in length in pMiDsf1 and between 26 and 900 bp in pMiDsf1-RI. Mock methylated (lanes 1 and 2) and untreated plasmids (lanes 3 and 4) are completely digested, whereas *in vitro* C-methylated pMiDsf1-*Met* and pMiDsf1-RI-*Met* (lanes 5 and 6) plasmids are left totally undigested confirming the completeness of the methylation raction.

2.3.1.2 Testing the replication capability of pMiDsf1-RI-*Met*

In order to have a replicating holo-methylated *Ds* element as control, it was necessary that pMiDsf1-RI-*Met* could still replicate in petunia cells in spite of CpG methylated replicon sequences. This was initially uncertain since the replication activity of other geminiviruses such as TGMV (Tomato Golden Mosaic Virus) (Brough *et al.*, 1992) and ACMV (African Cassava Mosaic Virus) (Ermak *et al.*, 1993), was reported to be inhibited by DNA C-methylation.

Therefore before proceeding with *Ds* excision analyses, the capability of pMiDsf1-RI-*Met* to replicate in petunia cells was checked. Aliquots of 10 µg of pMiDsf1-RI plasmid DNA propagated in the *Dam*$^{+}$-*Dcm*$^{+}$ *E. coli* strain DH5α were CpG methylated by *Sss* I-Methylase treatment or used for the preparation of the mock methylated pMiDsf1-RI-mock plasmid. pMiDsf1-*Met* and pMiDsf1-mock were produced under the same conditions. All methylated and mock-methylated plasmids were transfected into petunia protoplasts. The

Southern detection shown previously in Fig. 14, lane 6, indicates that *in vitro* cytosine methylation is maintained during culturing time in protoplasts, since no *Bsi* EI digestion was observed 30 hr after transfection in the non-replicating plasmid.

In order to look for replicative plasmid forms, total protoplast DNA was extracted 30 hr after transfection and subjected to *Mbo* I assay following the same procedure described in section 2.1.2.2.

The 1024 bp *Mbo* I digestion product formed in the presence of replication is not observed when the transfected plasmids are pMiDsf1-mock and pMiDsf1-*Met* (Fig. 18, lane 3 and 4). It is observed in protoplasts transfected with pMiDsf1-RI-mock (lane 1), and it is readily detected also when the transfected vector is pMiDsf1-RI-*Met* (lane 2) demonstrating that the plasmid can still replicate in spite of CpG methylation.

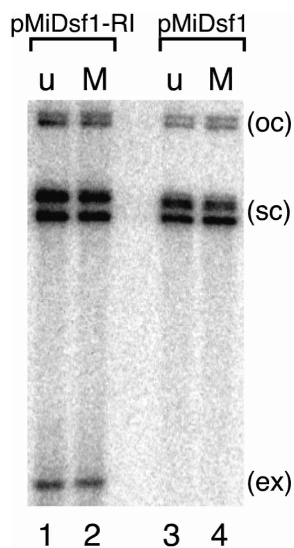


Fig. 18. Methylation does not impede replication of pMiDsf1-RI-*Met*. DNA was isolated from petunia protoplasts 30 hr after cotransfection with 10 µg TPase expression plasmid pNT600-10.ATG and 10 µg C-unmethylated (u) or -methylated (M) pMiDsf1-RI or pMiDsf1, digested with the *dam*-methylation sensitive enzyme *Mbo* I, electrophoresed, blotted and hybridized with the probe shown in Fig. 11A. oc: undigested, open circle forms of pNT600-10.ATG and the reporter plasmid; sc: undigested, supercoiled pNT600-10.ATG and reporter plasmid; ex: 1024 bp-*Mbo*I digestion fragment from the reporter plasmid.

2.3.1.3 Studies of the transposition competence of a holo-methylated *Ds*

The transposition competence of a CpG holo-methylated *Ds* in the absence and in the presence of replication was studied using the GUS-assay and DNA analyses:

2.3.1.3.1 GUS-assay

Petunia protoplasts were co-transfected with pNT600-10.ATG and 10 µg CpG methylated or unmethylated plasmids respectively. Plasmid DNA subjected to CpG methylation or mock-methylation treatment was originally propagated in the *Dam⁻-Dcm⁻* *E. coli* strain

JM110. This was to ensure that no factor other than DNA CpG methylation was responsible for the end result. After ca. 30 hr protoplasts were subjected to GUS-staining. The experiment was repeated several times; the average values of four comparable petunia assays are shown in Tab. 3. Each value corresponds to the average number of blue spots from two filters.

Tab. 3. GUS-assay of protoplasts transfected with methylated and mock-methylated *Ds* reporter plasmids. Petunia protoplasts were co-transfected with 10 µg pNT600-10.ATG and 10 µg C-methylated or mock-methylated reporter plasmids. 30 hr after transfection, protoplasts were spread on nitrocellulose filters and GUS-stained. Each value represents the average number of protoplasts counted on two filters. The results of four independent experiments are shown.

Ds excision events of holo- and non-methylated *Ds* elements in petunia protoplasts				
	number of GUS-positive cells			
	Exp. I	Exp. II	Exp. III	Exp. IV
pMiDsf1-*Met*	2	1	2	1
pMiDsf1-RI-*Met*	147.5	120	266	185
pMiDsf1-mock	255	170	145	143.5
pMiDsf1-RI-mock	448	384	310	325

In the absence of methylation, *Ds* can excise from both replicating and non-replicating plasmids and, as expected, with higher frequencies from the replicating pMiDsf1-RI-mock vector.

In the presence of methylation, *Ds* excision is observed only from the replicating pMiDsf1-RI-*Met*. The number of blue protoplasts observed in this case is lower in comparison to that observed for the mock-methylated plasmid control pMiDsf1-RI-mock. This is probably due to a delay in the first transposition events as a consequence of the initial inhibition of replication activity by C-methylation, as reported in the other geminiviruses TGMV (Brough *et al.*, 1992) and ACMV (Ermak *et al.*, 1993). In this reports, hypermethylation resulted in the reduced accumulation of viral DNA in transfected cells. Both the transcriptional inhibition of viral genes necessary for replication and an altered interaction between the replication complex and methylated viral DNA, are possibly involved in the retardation of the replication activity. Moreover, a weaker signal at the

1024 bp digestion product in Fig. 18 corresponding to pMi*Ds*f1-RI-*Met* (lane 2) seems in accordance with this explanation.

These results are in agreement with the model proposed by Wang and colleagues: only in the presence of replication is the transposition capacity of fully methylated *Ds* restored.
However, the low number of blue protoplasts observed in the case of pMiDsf1-*Met* can be explained, not only as a consequence of a lower transposition efficiency of *Ds*, but also as an effect of the lack of GUS gene expression due to the C-methylation of its own promoter. By transfection of protoplasts with fully methylated and mock-methylated pNT150 positive control (Becker *et al*., 1992), where no *Ds* element is inserted in the GUS gene, the expression of the GUS gene is decreased at least by a factor of 10^2.
Therefore, a DNA analysis has to be performed to study the behaviour of the *Ds* element in pMiDsf1-*Met*.

2.3.1.3.2 DNA analysis

In order to conclusively determine whether *Ds* also excises from methylated pMiDsf1, a PCR analysis was performed on total DNA extracted from protoplasts 30 hr after transfection. Using the same PCR primer pair as for the footprint analysis (section 2.2.2), the empty *Ds* donor site (300 bp excision product), the *Ds*-containing donor site (1000 bp product), and a 250 bp fragment from plasmid pNT150 were co-amplified. pNT150 lacks the *Ds* insertion in the GUS gene and was added in each reaction tube (1 pg) as a control. 150 ng of total protoplast DNA was used as template and the same selective PCR cycles described in section 2.2.2 were choosen for this study. They condition a strong underrepresentation of the *Ds*-containing donor site. PCR products were visualized by gel blot hybridization and quantified. A DNA fragment corresponding to the 300 bp excision product was used as probe for Southern detection.
With methylated, non-replicating pMiDsf1 (Fig. 19A, lane 4), an extremely weak signal of the empty donor site is detectable. Complete absence of methylation (lane 3) or replication (lane 2), restore the transposition capacity of the element, confirming the results observed in the GUS-assay. The weaker PCR signal detected by the methylated pMiDsf1-RI plasmid (lane 2) in comparison to its unmethylated form (lane 1) is in agreement with the initial inhibition of the plasmid replication activity by C-methylation of viral replicon sequences.

In Fig. 19A, the areas used for the quantification of the signals corresponding to the 300 bp and 250 bp PCR products, in mock-methylated and methylated pMiDsf1 respectively are indicated with a frame. Fig. 19B shows the intensity of radiolabel in gel areas R1, R2, R3 and R4 from which the respective background was subtracted. Ratios ε=11 and δ=1.1 (correction factor) are the outcome of (R1-B1)/(R2-B2) and (R4-B2)/(R3-B1), respectively (Fig. 19C). According to these values, CpG methylation reduces *Ds* excision by at least a factor of 12 (ε×δ).

In summary, these results prove that C-methylation of a *Ds* element severly inhibits transposition, and that this inhibition is overcome by replication.

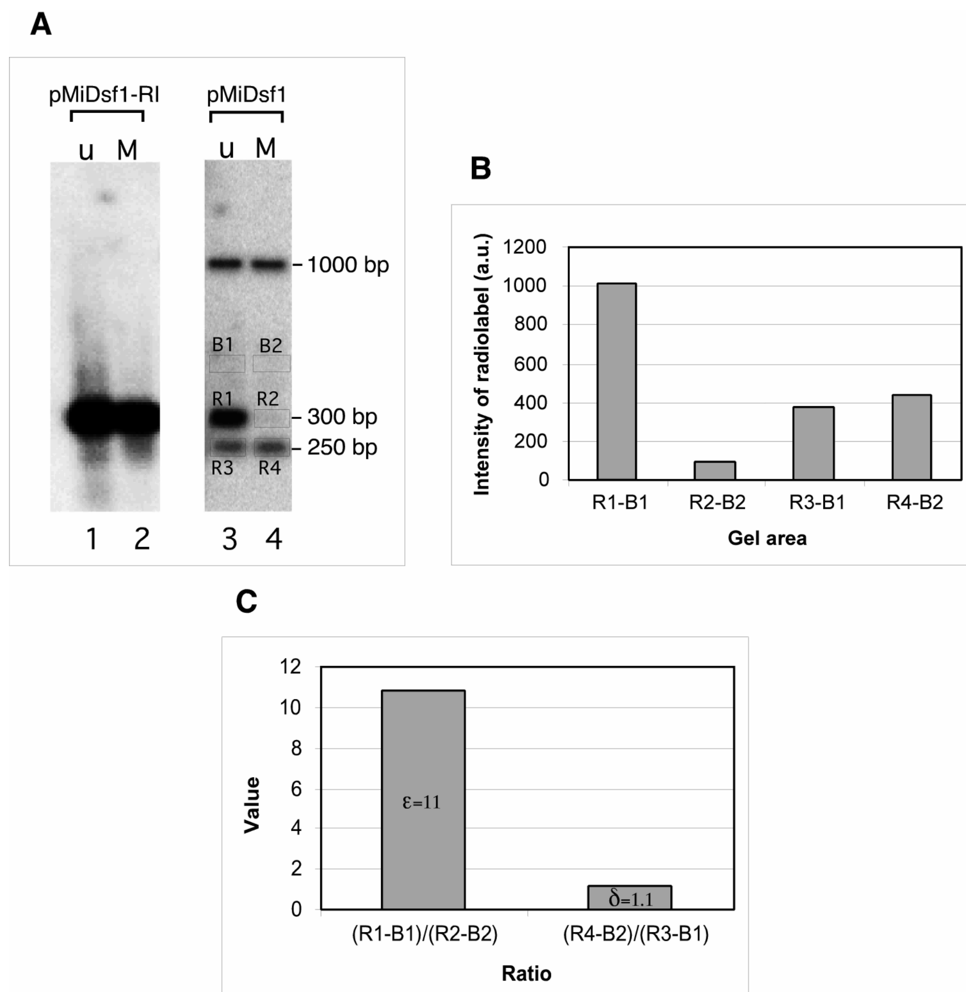


Fig. 19. PCR analysis of *Ds* excision from CpG methylated and unmethylated pMiDsf1 and pMiDsf1-RI plasmids. **A)** DNA was extracted from protoplasts 30 hr after co-transfection with 10 µg TPase expression plasmid pNT600-10.ATG and 10 µg C-unmethylated (u) or methylated (M) pMiDsf1-RI or pMiDsf1. 150 ng of total protoplast DNA were supplemented with 1 pg pNT150 and subjected to

PCR using *Ds*-flanking primers and selective PCR conditions. The PCR products corresponding to the empty *Ds* donor site (300 bp excision product), the *Ds*-containing donor site (1000 bp product), and the 250 bp fragment from plasmid pNT150, were analysed by gel blot hybridization using the empty donor site fragment of 300 bp as probe. The intensitiy of radiolabel in R1, R2, R3, R4, B1 and B2 gel areas was determined. **B)** Quantification of the intensity of radiolabel (arbitrary units, a.u.) in gel areas R1, R2, R3, and R4. B1 and B2 were used as background and subtracted from band signals R1/R3 and R2/R4, respectively. **C)** According to signal intensities, ratios ε=(R1-B1)/(R2-B2)=11 and δ=(R4-B2)/(R3-B1)=1.1 were calculated. Finally, CpG methylation reduces *Ds* excision by at least a factor of 12 ($\varepsilon \times \delta$).

2.3.2 Hemimethylation determines the transposition competence of the *Ds* elements

The model for *Ac*/*Ds* chromatid selectivity proposed by Wang *et al.* (1996) predicts that replication restores the transposition capability of a holomethylated element and that only one of the two different transiently hemimethylated *Ds* elements formed after replication is competent for transposition. In the previous experiments it has been shown that replication restores the transposition from a holomethylated plasmid. Nevertheless it was impossible to distinguish whether this restoration was caused by the hemimethylated element formed after one round of replication, or an unmethylated *Ds* after subsequent rounds of replication, or both.

In order to examine in a rigorous way the specific transposition competence of the hemimethylated states of the *Ds* transposon, a set of novel *Ds* reporter plasmids were constructed and tested *in vivo* in plant cells.

2.3.2.1 Preparation of "hemimethylated-*Ds*" constructs

An experimental procedure was established for the preparation of *Ds* reporter plasmids in which either the bottom or the top strand of the *Ds* element and not the flanking plasmid sequences, were CpG methylated. In this way, methylation did not interfere with GUS-gene expression.
Fig. 20 illustrates the strategy followed for the construction of "*Hemi-Ds*" constructs. The intermediate DNA forms generated in the most important steps during the entire procedure, are shown by ethidium bromide staining. The number on the bottom right corner of each gel indicates the procedure step to which the electrophoresis is referred. Arrows beside or inside the gels point at the intermediate DNA forms produced.
As a first step (Fig. 20, step (1)), the M13 f1 origin of replication was inserted into the *Ds* reporter plasmids for the preparation of phagemid single stranded DNA. Reporter plasmids

were transformed into the *E. coli* strain NM552, and after infection of the cells with M13KO7 helper phage, single stranded plasmids were produced and isolated. Fully methylated, biotinylated *Ds* element, amplified in the presence of ^{5m}dCTP and one biotinylated primer in the reaction mixture, was generated by PCR (step (2)). After purification by gel filtration, the PCR product was phosphorylated by treatment with T4 polynucleotide kinase and bound to magnetic streptavidine coated beads (step (3)). The phosphorylation reaction was tested by over night self-ligation of the *Ds* PCR product. The 1.4 kb ligation product expected in the absence of the phosphate group on the non-biotinylated *Ds* end was not observed. Streptavidine beads were incubated in NaOH after adsorption of the phosphorylated element and the non-biotinylated, phosphorylated and C-methylated *Ds* single-strand was eluted (step (4)). To prevent eventual degradation of the single stranded DNA by excessive incubation in NaOH solution, the basic eluate was immediatly neutralized by the addition of HCl. After purification by gel filtration, the *Ds* single strand was annealed to the complementary, unmethylated phagemid single stranded DNA in a molar ratio 2:1 (step (5)). At this ratio, determined by several test hybridizations, the optimal yield of hybridization product was achieved; the phagemid single stranded DNA was almost completely incorporated in the *Ds*/phagemid hybrid. Increasing amounts of *Ds* single strands in the hybridization solution did not improve the yield of hybridization complex anymore. The hybridization product migrates slightly slower than the single stranded DNA in 0.7% agarose gels.

The hybridization product was desalted and the residual single-stranded plasmid sequences were filled-in with *Pfu* DNA polymerase (step (6)). The filled-in product (lane 2), according to its relaxed structure, migrates on agarose gels like the relaxed form of the same plasmid isolated from *E. coli* (lane 1). Each filling-in reaction contained approximately 60 ng of hybridization product as template, with a final yield of filled-in plasmid of ca. 40 ng. This yield decreased to approximately 18-20 ng after the further purification steps. For the preparation of 3 µg of "*Hemi-Ds*" plasmid DNA (as explained below, in this experimental part petunia protoplasts were transfected with 3 µg of each *Ds* reporter plasmid instead of the standard 10 µg), at least 160 unique filling-in reactions were necessary per plasmid.

After purification of the final filled-in product by phenol extraction and ethanol precipitation, the remaining nick was closed by incubation with T4 DNA ligase (step (7)). Aliquots of 0.3 µg plasmid DNA were treated over night at 16°C with 2 units of T4 DNA

Ligase. The ligation product was finally extracted twice with phenol/chloroform and purified by ethanol precipitation. The DNA was resuspended in TE buffer.

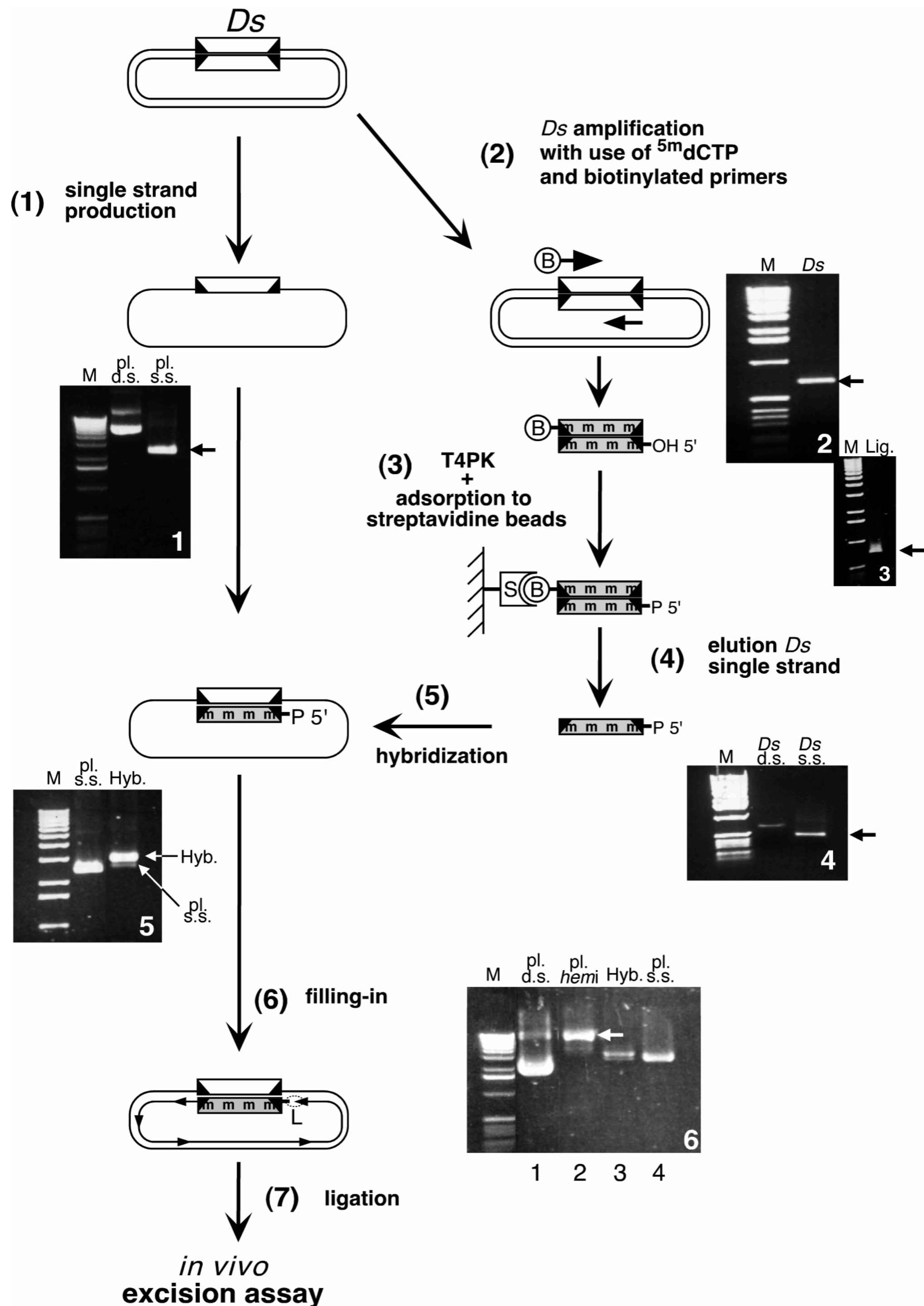


Fig. 20. Procedure for the construction of "*Hemi-Ds*" plasmids. For steps 1, 2, 4, 5 and 6, an ethidium bromide stained gel of the DNA form generated is shown. Arrows beside or inside the gels point at the DNA intermediate formed in each step. **M**: molecular weight marker. **(1)** Single stranded DNA

production from a *Ds* reporter plasmid. On 0.8% agarose gels, the single stranded plasmid (pl. s.s.) migrates faster than the supercoiled and relaxed double stranded plasmid forms. **(2)** A biotinylated, fully CpG methylated *Ds* element (0.7 kb) was prepared by PCR amplification. In the PCR reaction mixture, dCTP was replaced by ^{5m}dCTP and one of the two primers was biotinylated. **(3)** The PCR product was phosphorylated by treatment with T4 polynucleotide kinase (T4PK) and adsorbed to streptavidine beads. The phosphorylation reaction was verified by over-night self ligation of the element (Lig.). No 1.4 kb ligation product was observed, as expected by a incomplete phosphorylation reaction. **(4)** The single stranded phosphorylated and C-methylated *Ds* element (*Ds* s.s.) was separated from its complementary strand bound to the beads by NaOH treatment, and finally eluted. Also in this case the single stranded DNA migrates faster than the double stranded DNA (*Ds* d.s.) on agarose gels (1%). **(5)** Single stranded, unmethylated *Ds* reporter plasmid was hybridized with single stranded, methylated *Ds* element in a molar ratio 1:2. The formation of the hybridization product (Hyb.) was verified by gel electrophoresis: in a 0.7% agarose gel, the hybridization product migrates slightly slower than the single stranded form of the plasmid (pl. s.s.). **(6)** The plasmid single strand sequences remaining on the hybridization product are filled-in using the *Pfu* DNA polymerase. The final construct (pl. *hemi*, lane 2) migrates at the same velocity as the relaxed form of the same plasmid propagated and isolated from *E. coli* (pl. d.s., lane 1). Hyb.: hybridization product before *Pfu*-filling-in reaction (lane 3); pl. s.s.: single stranded plasmid (lane 4). **(7)** The residual single strand nick, still present at one *Ds* border was closed with T4 DNA ligase.

Before transfection of plant cells with the newly constructed "*Hemi-Ds*" plasmid DNA, hemimethylation of the *Ds* element in the constructs was confirmed by restriction enzyme analysis of the final product (Fig. 21). Only few enzymes are hemimethylation sensitive; moreover, it was essential to find one with this property that cuts at least once within the *Ds* element. The enzyme choosen having such characteristics was *Alu* I. This enzyme is C-hemi-methylation sensitive, it cuts once in the *Ds* element, in the subterminal region of *Ds* 5' end (no other enzyme was available which cuts more than once in the *Ds*) and 26 times in the surrounding plasmid sequence. As described in Fig. 21A, a 2483 bp fragment is expected in all plasmids in the presence of hemimethylated *Ds*. The two digestion products of 700 and 1783 bp, expected in the absence of methylation, should not be observed. In Fig. 21B, the digestion pattern in the absence of methylation is illustrated for each "*Hemi-Ds*" construct produced (lane 1 in each gel). The figure demonstrates that the 2483 bp fragment is formed only in the "*Hemi-Ds*" constructs. No 1783 bp digestion product is detected; no statement can be made about the 700 bp long fragment since at least 3 other fragments around 700 bp in length are generated by *Alu* I digestion in the rest of the plasmid and can not be distinguished on the gel.

These results confirm that the constructed "*Hemi-Ds*" plasmids are hemimethylated at the *Ds* element, and the hemimethylation is restricted to the element. The rest of the plasmid is properly digested by *Alu* I restriction.

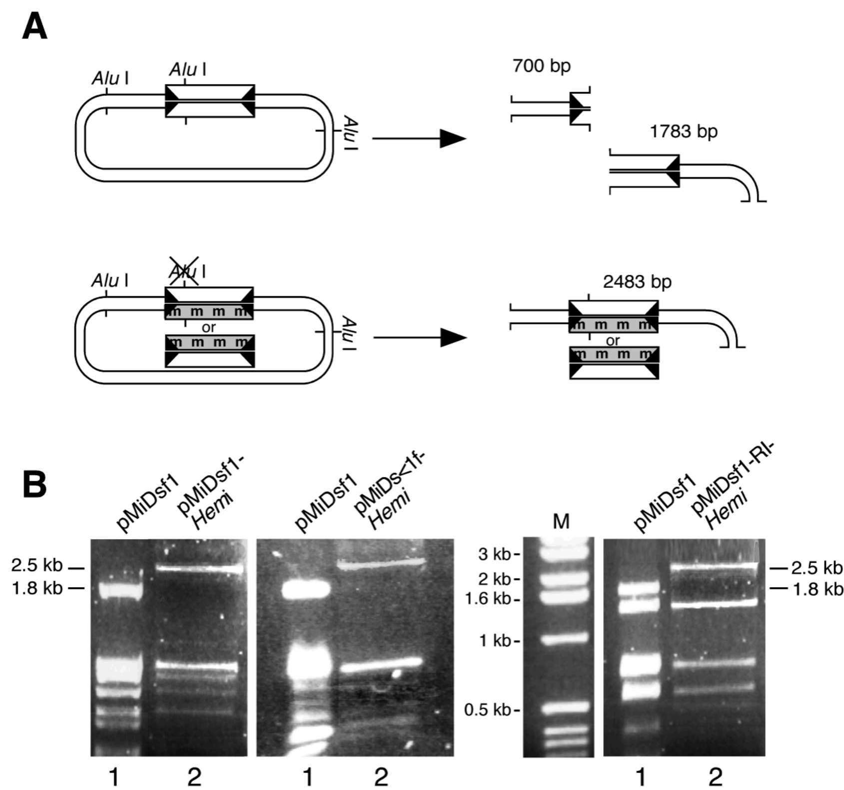


Fig. 21. *Alu* I digestion control of "*Hemi-Ds*" constructs. A) Plasmid DNA *Alu* I restriction digestion fragments, expected in the absence and in the presence of hemi-methylation at the *Ds* element. In the absence of methylation, two fragments of 700 and 1783 bp are generated. In the presence of an hemimethylated *Ds* a unique fragment of 2483 bp should appear. **B)** *Alu* I restriction digestion of pMiDsf1-*Hemi*, pMiDs<1f-*Hemi* and pMiDsf1-RI-*Hemi* (lane 2 of each stained gel) and the relative unmethylated forms (lane 1 of each stained gel). M: size marker. The 2483 bp digestion product is only generated by the "*Hemi-Ds*" constructs, and no 1783 bp fragment is observed.

2.3.2.2 GUS-staining studies on "*Hemi-Ds*" constructs

A first transfection experiment was performed with the constructs pMiDsf1-*Hemi* (lower-strand hemimethylated *Ds*) and pMiDs<1f-*Hemi* (top-strand hemimethylated *Ds*), prepared following the above procedure. The non-replicating pMiDsf1 and pMiDs<1f plasmids (the latter carries the M13 f1 origin of replication in opposite orientation than pMiDsf1) were used for the production of single stranded phagemid DNA as outlined in Fig. 22 in step (1). In step (2) the primer pairs bDs2-mDs3 and Ds2.II-bDs3 were employed for the preparation of pMiDsf1-*Hemi* and pMiDs<1f-*Hemi* respectively and ^{5m}dCTP was included in the reaction mixture. Petunia protoplasts were co-transfected with 10 µg of pNT600-10.ATG and 3 µg of either pMiDsf1-*Hemi* or pMiDs<1f-*Hemi*. After 30 hr protoplasts were subjected to GUS-staining. The results are shown in Table 4. The top-strand

hemimethylated *Ds* element achieves an approximately 5-fold higher excision frequency than the bottom-strand hemimethylated *Ds* (Tab. 4, Exp. I).

Tab. 4. GUS-assay of protoplasts transfected with methylated and mock-methylated *Ds* reporter plasmids. Petunia protoplasts were co-transfected with 10 µg pNT600-10.ATG and 3 µg hemimethylated or mock-methylated reporter plasmids. 30 hr after transfection, protoplasts were spread on nitrocellulose filters and GUS-stained. Each value represents the average number of protoplasts counted on three filters. The results of three independent experiments are shown.

Transposition competence of hemimethylated *Ds* elements in petunia protoplasts			
	number of GUS-positive cells		
	Exp. I	Exp. II	Exp. III
pMiDsf1-*Hemi*[b]	18	13	14
pMiDs<1f-*Hemi*[c]	90	90	105
pMiDsf1-mock[d]	n.d.	110	95
pMiDsf1-RI-*Hemi*[e]	n.d.	90	100

[b] bottom strand-hemimethylated *Ds*, non-replicating
[c] top strand-hemimethylated *Ds*, non-replicating
[d] mock-methylated *Ds*, non-replicating
[e] bottom strand-hemimethylated *Ds*, replicating

If the decrease of transposition capacity of the *Ds* element in pMiDsf1-*Hemi* is really due to its methylation status, then normal transposition frequencies should be restored either by complete absence of methylation or by replication. Two control plasmids were prepared using the same procedure employed for pMiDsf1-*Hemi* and pMiDs<1f-*Hemi* construction. The first control plasmid, pMiDsf1-mock, can not replicate in petunia protoplasts and its *Ds* element is unmethylated. For the preparation of this control, the plasmid pMiDsf1 provided the single stranded phagemid in step (1) and the primer pair bDs2-Ds3 and dCTP were included in the PCR mixture in step (2). The second control plasmid, pMiDsf1-RI-*Hemi*, can replicate in petunia cells and carries a bottom-strand hemimethylated *Ds* element (like in pMiDsf1-*Hemi*). For the preparation of this control, the plasmid pMiDsf1-RI provided the single stranded phagemid in step (1) and primer pair bDs2-mDs3 and ^{5m}dCTP were included in the PCR mixture in step (2).
Two additional protoplast transfection assays were performed; each time protoplasts were transfected with 3 µg of freshly prepared DNA construct. The outcome of the GUS-staining is shown in Tab. 4, Exp. II and III. It confirms the data from the first experiment: in pMiDs<1f-*Hemi*, methylated on the top strand, the *Ds* element excises approximately

6.3-fold more frequently than in pMiDsf1-*Hemi*, where the bottom strand is methylated. Normal transposition frequencies of pMiDsf1 are restored in the absence of methylation (pMiDsf1-mock) and in the presence of replication (pMiDsf1-RI-*Hemi*). With the latter plasmid, the intensity of the blue staining of each protoplast was on average higher than with pMiDsf1-mock, corroborating its replication activity.
The initial hemimethylation status of *Ds* in pMiDsf1-RI-*Hemi* supposedly induces a delay in the first transposition event. This is probably the reason why the higher transposition frequency expected for a replicating plasmid in comparison with a non-replicating one like pMiDsf1-mock, was not observed.

2.3.2.3 Occupation of transposition-competent *versus* -incompetent *Ds* elements with TPase

Ac/*Ds* elements contain multiple short TPase binding motifs in both ends which contain a AAACGG sequence (Kunze and Starlinger, 1989; Becker and Kunze, 1997) (Fig. 22A). DNA-binding studies with synthetic oligomers of these motifs have shown that *in vitro* the *Ac* TPase protein binds selectively to such sites that are hemimethylated on the bottom strand, whereas a 5-methylcytosine on the top strand inhibits TPase binding (Kunze and Starlinger, 1989) (introduction section 1.3.4.1).
To this end it remained an open question whether *Ac* TPase binding affinity to these synthetic, hemimethylated concatamers reflects the binding properties to hemimethylated transposon ends (i.e. the TPase binding motifs in their native sequence environment). Therefore the *in vitro* binding properties of the TPase to hemimethylated, transposition-competent or -incompetent *Ds* ends were analysed. The complete *Ds* 5' and 3' ends and the three TPase binding site clusters "I", "II" and "III" from the 5' end (Fig. 22A) were synthesized in the unmethylated ("u"), holomethylated ("M"), and both alternate hemimethylated states (bottom strand methylated: "bm" and top strand methylated: "tm").
Hemimethylated *Ds* ends were prepared by combinatorial hybridization of complementary methylated and unmethylated single stranded *Ds* DNA followed by *Dra* I digestion of the hybridization product. *Dra* I, a C-methylation resistant restriction enzyme, cuts once within the 700 bp *Ds* element to produce a 300 bp long 5' end and a 400 bp long 3' end. Methylated and unmethylated *Ds* single strands were produced as outlined in steps (2), (3) and (4) of Fig. 20, (without phosphorylation treatment in step (3)) for the construction of "*Hemi-Ds*" plasmids. Either ^{5m}dCTP or dCTP were used for the PCR amplification of the

Ds element in step (2). Holomethylated and unmethylated *Ds* ends were prepared by PCR amplification of the *Ds* element in the presence and in the absence of ^{5m}dCTP in the reaction mixture. The PCR product was then digested with *Dra* I.
The 5' end segments I, II and III in their unmethylated, holomethylated and hemimethylated forms, were generated by combinatorial hybridization of complementary methylated or unmethylated synthetic oligonucleotides corresponding respectively to positions 30-96, 106-158, and 168-242 of the *Ac* sequence.

The *in vitro* binding affinities of each DNA form to an *E. coli* synthesized *Ac* TPase$_{103-465}$ (Feldmar and Kunze, 1991) protein fragment were determined by gel shift assay (Fig. 22 B). This fragment is a N- and C-terminal truncated *Ac* TPase protein containing the bipartite DNA binding domain that extends from residues 159 to 206 (Becker and Kunze, 1997). Its binding properties to synthetic binding sites are shown to be similar to those of the wild-type TPase$_{1-807}$ (Feldmar and Kunze, 1991).
The diffuse appearance of the shift bands shown in Fig. 22B is characteristic for TPase/DNA complexes (Kunze and Starlinger, 1989; Feldmar and Kunze, 1991; Becker and Kunze, 1997). Presumably this is caused by the presence of heterogenous TPase oligomers in the protein preparation, leading to an heterogeneous composition of the TPase/DNA complexes (Feldmar and Kunze, 1991).
TPase binds efficiently to the unmethylated *Ds* 3' end (Fig. 22B, lane 20). Remarkably, TPase affinity is even increased when the 3' end fragment is hemimethylated on the top strand (Fig. 22B, lane 19). This is the transpositionally competent state (Table 4, pMiDs<1f-*Hemi*). In this hemimethylation state, 12 out of 14 subterminal TPase binding site motifs are able to bind TPase protein (Fig. 22C, tm). In the alternate hemimethylation state, which correlates with very low transposition activity, the 3' end is only very weakly bound (Fig. 22B, lane 18). This is in accordance with the assumption that in this state, TPase can recognize only two widely separated TPase binding site motifs. When the 3' end is methylated on both strands, it is bound by TPase only in traces (Fig. 22B, lane 17).

At the 5' end the situation is different. As is the case with 3' sequences, holomethylation prevents TPase binding (Fig. 22B, lane 1). However, both hemimethylated target DNAs are bound with similar efficiencies (lanes 2-3). The unmethylated state is recognized with a slightly higher affinity than the hemimethylated DNAs (lane 4).

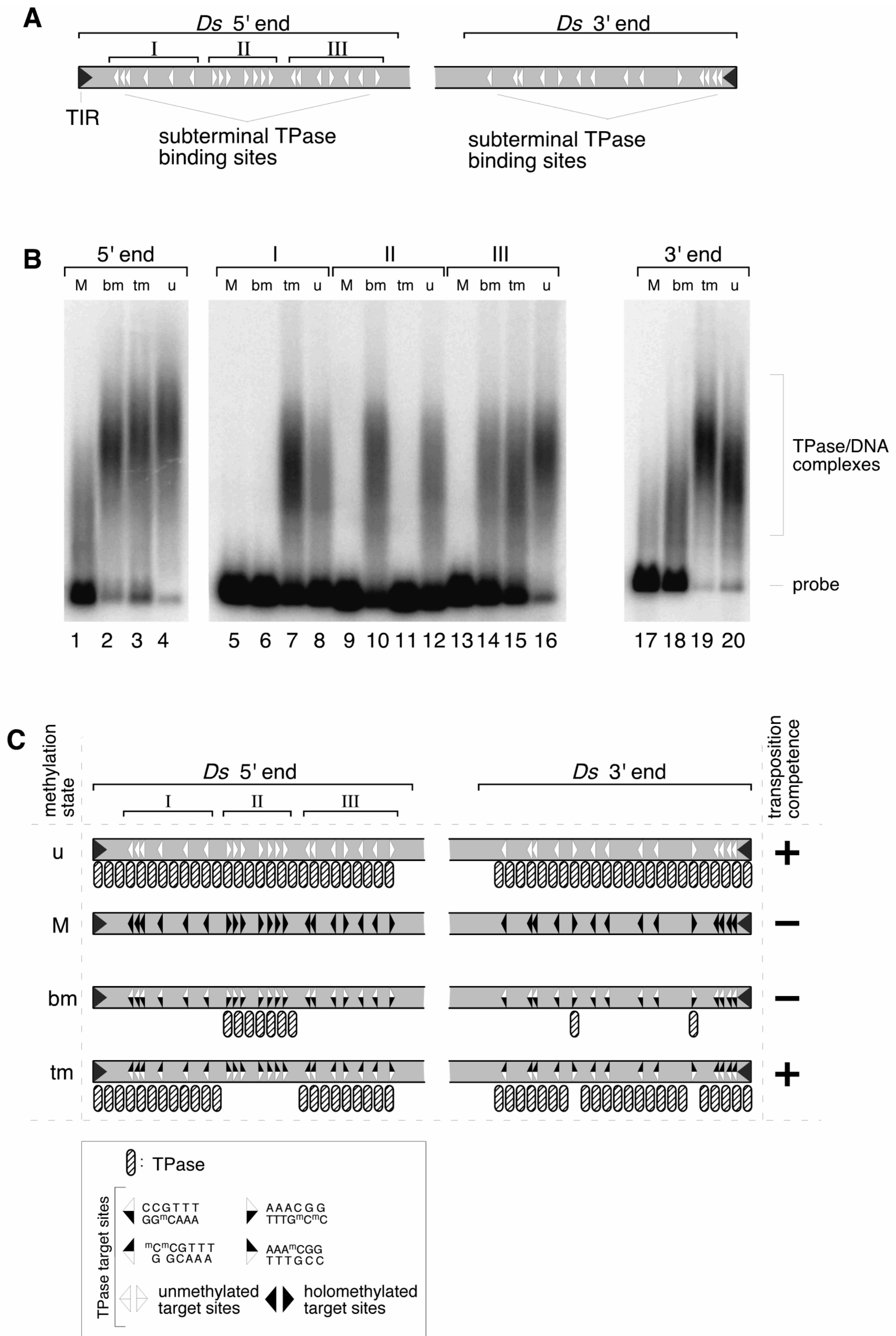


Fig. 22. Gel mobility shift assay of TPase binding to differentially methylated *Ds* end-fragments. A) Schematic of the *Ds* ends. The *Ds* 5' and 3' subterminal regions (250 bp and 200 bp of the transposon termini, respectively) comprise the TPase binding sites. The three 5' end segments 'I', 'II', and 'III' correspond to positions 30-96, 106-158, and 168-242 on the *Ac* sequence. The distribution of TPase

binding sites (open triangles) is drawn approximately to scale. **B)** Gel mobility shift assays with the *Ds* end fragments in different methylation states and the $TPase_{103-465}$ protein. u: unmethylated; M: holomethylated; bm: bottom strand-methylated; tm: top strand-methylated. Free probe DNA migrates at the bottom of the gels. **C)** Model for the methylation-dependent TPase binding to the *Ds* 3' end and 5' end fragments. The proposed occupation of the *Ds* terminal fragments with TPase dependent on the methylation state, according to the gel shift assays shown in panel B and binding studies with synthetic target site oligomers (Kunze and Starlinger, 1989; Becker and Kunze, 1997) are shown. The four different methylation states are designated at left as in panel B. The transposition competence of the respective (hemi)methylated transposons are indicated at the right.

The 5' end contains three binding site clusters ("I", "II", and "III"; Figure 22A) and the central cluster "II" has opposite orientations of TPase binding sites relative to clusters "I" and "III". Therefore the occupation of each of these clusters with TPase was individually tested.

The gel shift assays show that in the unmethylated state, clusters "I" and "II" are moderately well bound (lanes 8 and 12) and cluster "III" is more efficiently complexed (lane 16). Methylation on both DNA strands completely inhibits TPase binding to all three fragments (lanes 5, 9, 13). The terminal cluster "I" is most efficiently bound in the transpositionally competent, top strand-hemimethylated state (lane 7). The innermost cluster "III" is also recognized in this state (lane 15), whereas the central cluster "II" is not bound at all (lane 11). In the transpositionally inactive, bottom strand-hemimethylated state, the 5' terminal cluster I is not recognized by TPase (lane 6), whereas cluster II is efficiently bound (lane 10) and cluster III is weakly complexed (lane 14).

In summary, these experiments demonstrate that (hemi)methylation of the subterminal TPase binding sites is a *cis*-acting determinant of transposition activity of *Ds* elements. In the active state, both *Ds* ends have a high affinity for the *Ac* TPase protein, whereas the inactive state correlates with very reduced TPase binding to the 3' end and to the 5' end TPase binding site cluster I.

These results are supported by previous data of mutational analysis on TPase subterminal binding sites at both *Ac*/*Ds* ends (Coupland *et al*., 1989; Chatterjee and Starlinger, 1995; Weil and Kunze, 2000), outlining the crucial role of the 5' end and 3' end distal binding sites on the transposon excision mechanism. Figure 22C shows a model of the occupation of the *Ds* terminal fragments with TPase molecules dependent on the methylation state, according to the gel shift assays shown in panel B and binding studies with synthetic target site oligomers (Kunze and Starlinger, 1989; Becker and Kunze, 1997).

3 DISCUSSION

3.1 Replication as a transposition regulatory factor independent of DNA methylation

3.1.1 In monocots the replication-dependence of *Ac/Ds* transposition is more pronounced than in dicots

Until now, *in vivo* studies on the effect of DNA replication on *Ac/Ds* elements excision frequency, in the absence of methylation, have been performed exclusively in monocot species (Laufs *et al.*, 1990; McElroy *et al.*, 1997; Wirtz *et al.*, 1997). *Ac/Ds* elements excision from extrachromosomal reporter vectors was analysed also in these works. *Ac/Ds* reporter plasmids, replicating and non-replicating in plant cells were constructed. Replicon sequences were provided in all cases by the Wheat Dwarf Virus (WDV), a geminivirus infecting various cereal species. Replicating and non-replicating *Ac/Ds* reporter plasmids were transfected into the cells of several cereals such as wheat (Laufs *et al.*, 1990), rice (Laufs *et al.*, 1990), barley (McElroy *et al.*, 1997) and maize (Laufs *et al.*, 1990; Wirtz *et al.*, 1997). In two cases (McElroy *et al.*, 1997; Wirtz *et al.*, 1997) replicating and non-replicating *Ds* reporter plasmids were employed and the transposase was provided *in trans* by a cotransfected TPase expression plasmid. Lauf and colleagues (Laufs *et al.*, 1990) worked with replicating and non-replicating reporter plasmids containing an autonomous *Ac* and no TPase expression plasmid was cotransfected. The excision of the *Ac/Ds* elements was investigated either by PCR (Laufs *et al.*, 1990; Wirtz *et al.*, 1997) or by PCR and GUS-staining (McElroy *et al.*, 1997) analysis.

Importantly, *Ds* excision was detectable only in the presence of replication. In maize, Wirtz and coworkers quantified the transposition enhancement factor and replication was found to improve *Ds* transposition by at least 10^5-fold.

In the present work it has been demonstrated that *Ds* does not need replication to be taking place in order to transpose. This was done in the dicot *Petunia hybrida* using an *in vivo* experimental system comparable to that employed in monocot species. Significant *Ds* transposition activity could be detected irrespective of the replication status, as demonstrated by PCR and by GUS-staining analysis.

Ds reporter plasmids, similar to those non-replicating used in this work, were previously transfected also in other dicot species, such as *Nicotiana plumbaginifolia*, tobacco and parsley (Houba-Hérin *et al.*, 1990; Houba-Hérin *et al.*, 1994b; R. Kunze unpublished). *Ds* excision was also observed in these cases.

What could account for the different behaviour of *Ac*/*Ds* in relation to DNA replication dependence observed in monocots and dicots? Fundamentally three possible explanations can be suggested:

a) A methodological explanation: **(i)** the analyses performed to reveal *Ds* excision events in monocots were not sensitive enough to detect the events in the absence of replication. In monocot works, a PCR approach was always employed to reveal *Ds* excision events. Only in one case, in barley (McElroy *et al.*, 1997), was an additional GUS-staining detection used (also McElroy and colleagues worked with *Ds* reporter plasmids where the GUS gene expression was interrupted by a *Ds* element). In contrast to what was observed in the petunia transfection system, both methodologies proved to be inadequate to detect *Ds* excision in the absence of replication. These groups used PCR conditions that were not optimized for the detection of rare *Ds* excision sites among a huge surplus of non-excised elements. The GUS-assay was performed in barley on scutellar tissues, co-transfected by microbombardement with a TPase expression vector and the *Ds* reporter plasmid. The transfection efficiency in barley was always as low as 0.01%. By such low transfection efficiencies also the petunia transient assay employed in this work failed to reveal, by GUS-staining, excision events from a non-replicating *Ds* element. Standard transfection efficiencies were in petunia of 10%-15% **(ii)** *Ds* element excision from unreplicating *Ds* reporter plasmid could have been negatively influenced by plasmid sequence environment in monocot studies, so that rare excision events were not detected by the methods employed. In the petunia transfection system, the excision efficiency of a non-replicating *Ds* element is influenced by the plasmid sequence environment. The plasmid pMiDsf1-ΔRI (Results section 2.2.1.1) differs from pMiDsf1 only by the absence of the Luciferase gene, and this difference causes a 3- to 4-fold reduced excision frequency compared to pMiDsf1. *Ds* reporter constructs employed in monocots were quite different from those employed in petunia. For instance, either the vector consisted of the WDV genome where the the viral (+) strand ORFs were replaced by the *Ds* element, or bacterial vectors were used where the

WDV replication sequences were inserted in the *Ds* element. Non-replicating reporter plasmids carried mutated non-functional replicon sequences. To conclusively answer the question of what nature the difference in *Ds* transposition response between mono- and dicots it would be important to investigate the properties of the vectors I used in the petunia system in monocots as well.

b) It is conceivable that in monocots, non-replicating (geminivirus) extrachromosomal vectors or *Ac*/*Ds* elements alone are methylated immediately after transfection. As a consequence, no TPase would be expressed due to promoter inactivation and/or TPase can not bind the holomethylated transposon ends. Methylated cytosines become demethylated during replication, restoring TPase/DNA transposition competence. This would explain the "catalytic" function of replication. It is conceivable that monocots possess a *de novo* methylation system that specifically acts on transposable elements. In fact, most *Ac*/*Ds* elements in maize are heavily methylated at the transposase binding sites, whereas the flanking host DNA is unmethylated (Wang *et al.*, 1996; Wang and Kunze, 1998). Similar specific modifications occur in the *Spm* transposon system (Banks *et al.*, 1988). Interestingly, no evidence was found for an alterated state of *Ac* methylation in transgenic tobacco, petunia and Arabidopsis plants (Keller *et al.*, 1992; Ott *et al.*, 1992; Lawson *et al.*, 1994; Robbins *et al.*, 1994).
Consistently, in this work no *de novo* methylation of non-replicating plasmids was observed after transfection. This might explain why *Ds* transposition can occur in the absence of replication. However, it can not explain the transposition boost observed during replication.

c) *Ds* transposition could be regulated by cell-cycle specific factors other than DNA methylation and this cell-cycle dependence is much tighter in monocots than in dicots.
It is possible that a replication-specific TPase-activating cofactor is required in monocots, which in dicots either improves a transposition activity that was already initiated in interphase, or is not replication-specific at all. Instead it could be expressed constitutively in the cell, and other factors (i.e. chromatin structure) facilitate transposition during the replication.

Alternatively a cell cycle-dependent inhibitor of transposition is acting in monocots, which is removed as soon as S-phase initiates, allowing transposition to proceed. In dicots this inhibitory factor may be missing or rather ineffective.

Specific transposition repressors that regulate the actvity of endogenous *Ac*-related elements have already been described. Little is known about what these factors really are and what their regulatory role in transposition is. In *Antirrhinum majus* there is a locus that negatively regulates the transposition of the *Ac*-related *Tam3* transposable element (Carpenter *et al*., 1987). This locus, referred to as *Stabilizer* (*St*), is unlinked to the *Tam3* element, specifically interacts with *Tam3* and does not affect the mobility of the *En*/*Spm* family elements *Tam1* or *Tam2* (Harrison and Carpenter, 1973). It is suggested that *St* is a mutant derivative of *Tam3*, expressing an inhibitory mutant version of the *Tam3* TPase which with a high-affinity binding competes for TPase binding sites (Chatterjee and Martin, 1997).

The recently isolated *iae*1 and *iae*2 (increased *Ac* excision) loci in Arabidopsis possibly play a similar role as *St*. These loci, which are unlinked to *Ac*, are known to repress *Ac* transposition in transgenic Arabidopsis plants (Jarvis *et al*., 1997a; Jarvis *et al*., 1997b). The transposition of *Ac*-related *Tag1* and *Tam3* transposons also appears to be negatively influenced by the *iae* loci (Jarvis *et al*., submitted for publication). Mutations in *iae*1 and *iae*2, result in 550- and 70-fold increases in *Ac* excision frequency in cotyledons respectively. A biochemical characterization of these loci has not yet been performed and still unknown is the nature of their action.

Putative host factors, possibly also associated to an inhibitory activity on *Ac* transposition, are identified in maize nuclear protein extracts. They interact with both *Ac* transposon ends *in vitro* (Becker and Kunze, 1996). Most of their DNA binding motifs (GDTAAA) either overlap or are located in the vicinity of transposase binding sites (Kunze and Starlinger, 1989; Becker and Kunze, 1997). The long TIRs of the maize *Mu1* element contain a very similar sequence that is also bound by a maize nuclear protein (Zhao and Sundaresan, 1991; Benito and Walbot, 1997), though is not yet known if this is the same protein that binds *Ac*. It is not yet known what the involvement of these factors is in transposition. Mutated *Ds1* elements that are bound less efficiently by these host proteins only show a reduced rate of excision in a few cases (Gorbunova *et al*., 2000). Perhaps these factors are not involved in transposition. Nevertheless, since *Ds1* is an atypical *Ds* element whose homology to *Ac* is restricted to 13 bp of the 5' end and 26 bp of the 3' end (Sutton *et al*.,

1984; Gerlach *et al*., 1987), it might be that the host factor requirements of *Ds1* differ from those of other *Ds* elements.

Interestingly these nuclear factors are relatively abundant in endosperm compared with *Ac* TPase. Moreover, a DNA bending activity is probably associated with their function since a conspicuous amount of DNase I hypersensitive sites was observed between the protected regions (Becker and Kunze, 1996). These evidences raised the possibility that these factors modulate *Ac* transposase protein by blocking certain transposase binding sites. This would explain the increased transposition frequency during *Ac*/*Ds* replication, when the relatively open conformation of DNA around the replication fork is possibly no longer targeted by these modulators and TPase has free access to its binding sites.

These nuclear factors do not appear to be specific for maize. Similar modulators have been identified also in *Nicotiana sylvestris*, which recognize the same sequences within *Ac* termini (Levy *et al*., 1996). Whether such components are present also in petunia cells is unknown. In that case, one could imagine that the *Ac* transposition observed in petunia protoplasts in the absence of replication is consequent to a weaker activity (lower binding affinity, other cell factors interfering with binding......) of such modulators in petunia cells (and in general in dicots) than in maize, that would account for easier access of the TPase to the *Ac* termini. All these hypotheses have yet to be examined.

3.1.2 DNA conformational changes by the replication fork. A possible explanation of the transposition boost observed in dicots during replication

The excision efficiency of the *Ds* transposon was increased in petunia protoplasts by inserting a replication function into the reporter plasmid. Replication enhances *Ds* transposition by at least a factor of 7.5. However, only a 1.4-fold difference in plasmid copy number is observed between replicating and non-replicating *Ds* vectors after transfection (compared to a 10-fold excess observed in maize (Wirtz *et al*., 1997)). This means that only a fraction of the transfected plasmids containing the replicon replicate in the petunia cells. It has been described that the Rep protein transcriptionally represses its own gene (Sunter *et al*., 1993; Eagle *et al*., 1994), or it is toxic in petunia cells. This has been observed when Rep is expressed in other plants, in *E. coli*, *Agrobacterium* and *Rhizobium* (B. Gronenborn, personal communication), where rearranged or inactive mutant forms of the protein appear. Without these limiting factors for plasmid replication the

boost in *Ds* excision frequency in petunia transfection system would probably be even more pronounced.

At present it is only possible to speculate about the DNA methylation independent molecular mechanisms that connect DNA replication with *Ds* transposition.
The fact that a mutation in the Rep binding site (IR) abolishes the transposition boost (Results section 2.2.1.1) rules out the possibility that the stimulatory effect of replication is a consequence of stimulation by the direct interaction of the replicase with the *Ac* TPase or of an activation of a host cell accessory factor by Rep. Rather, it is conceivable that the conformational change of the transposon ends within the replication fork and/or the interaction with some replication specific factors facilitates the access of the TPase to its binding sites and the formation of the "transpososom complex".

3.1.2.1 DNA conformation and transposition mechanism

The conformational state of DNA during the replication process is modulated by many structural proteins, i.e. DNA bending and unwinding proteins. Interestingly, the transposition mechanism of many prokaryote transposable elements either requires or is modulated by such host-encoded DNA binding proteins, also in cases when transposition is not dependent on DNA replication.
Bacteriophage *Mu* needs the HU protein for an early step in *Mu* DNA transposition facilitating transposition complex assembly. MuA-transposase binding sites in the *Mu* left end are brought together by the bending action of HU (Craigie *et al.*, 1985; Surette *et al.*, 1987; Lavoie and Chaconas, 1996). In addition, integration host factor (IHF) may also be required (Surette and Chaconas, 1989) depending on the topological state of the DNA. This factor permits the regulation of transposition by repressors and ensures that *Mu* never inserts into itself (Segall *et al.*, 1994). HU and IHF proteins are small, DNA binding basic architectural proteins that modulate DNA geometry and topology and favour protein-protein and protein-DNA complex assembly during many cellular processes, especially DNA replication and DNA repair.
Three host proteins, IHF, Xis and Fis, are involved in integration and/or excision recombination of bacteriophage λ (Craig and Nash, 1984; Thompson *et al.*, 1987; Ball and Johnson, 1991). They all bind to specific sequences interspersed between integrase binding sites and induce dramatic DNA bending.

Tn10 efficiently transposes *in vitro* in the absence of any host factor (Chalmers and Kleckner, 1994), but *in vivo* IHF and HU modulate its transposition activity (Kleckner *et al.*, 1996). In particular, the tight binding of IHF to a specific consensus sequence causes a 140° bend in the DNA and thus promotes the formation in a precleavage step of a transposon folded intermediate during transpososom assembly (Sakai *et al.*, 2000). Interestingly, like *Ac*, *Tn10* is also known to transpose preferentially just after the DNA replication fork passes (Roberts *et al.*, 1985; Kleckner, 1990). However up to now, no correlation with IHF programmed action but only with DNA *dam*-methylation has been reported to account for the replication dependence.

Direct evidence for a role of DNA conformation determinants exists in eukaryots in the V(D)J recombination reaction, which is very tightly related to transposition (Roth and Craig, 1998) and stimulated by the high mobility group (HMG) proteins (van Gent *et al.*, 1997; Agrawal *et al.*, 1998) that are also important components of eukaryotic DNA replication. V(D)J recombination requires a pair of signal sequences with spacer lengths of 12 and 23 bp between the conserved heptamer and nonamer elements. The RAG1 and RAG2 proteins initiate the reaction by making double-stranded DNA breaks at both signals, and must thus be able to operate in these two different spatial arrangements. HMG1 and HMG2 stimulate cleavage and RAG proteins binding at the 23 bp spacer signal. DNA bending is therefore important for bridging the longer spacer. Moreover HMG1 and HMG2 greatly stimulate coupled cleavage when both signal sequences are present, suggesting that these protein also aid in the formation of a synaptic complex.
At present, no analogs of these factors acting in V(D)J recombination have been identified in the transposition of plant elements. In Drosophila a cellular major factor in *P* element transposition has been isolated and proven to be a Drosophila homologue of the 70 kDa subunit of the human Ku autoimmune antigene. This has been called inverted repeat binding protein (IRBP) (Rio and Rubin, 1988) and specifically recognizes the outer half of the 31 bp terminal inverted repeats of the *P* element. It appears to facilitate transposition and play an essential role in double strand break repair after the transposon has excised (Staveley *et al.*, 1995; Beall and Rio, 1996). Importantly, its homologue Ku binds free DNA termini in a sequence independent manner (Mimori and Hardin, 1986). Ku is implicated in immunoglobulin V(D)J recombination (affecting both signal and coding joint formation) (Taccioli *et al.*, 1994), DNA repair, transcription and DNA replication. In

addition, Ku possesses ATPase and DNA helicase activities (Cao *et al*., 1994; Tuteja *et al*., 1994), making IRBP a possible candidate for a DNA unwinding protein similar to the activity of prokaryotic host factors. To date, repeated attempts by several laboratories to identify Ku70, Ku80 or DNA-PK_{cs} homologues in plants have been unsuccessful (Kunze and Weil, 2000, submitted for publication).

Possibly a cell-cycle programmed action of the above-mentioned DNA structure determinants accounts for the observed relationship between DNA replication and *Ac*/*Ds* transposition. This would be accomplished either by protein-DNA (modifying DNA structure and increasing accessibility of TPase to binding sites) or protein-TPase (improving TPase catalytic activity) interactions. Recently, HMG proteins have been identified in the nuclei of plant cells, both in dicots and monocots, that might perform such architectural functions, but no contribution to any transposition activity has been shown yet (Spiker, 1984; Moehs *et al*., 1988; Grasser, 1995; Grasser, 1998). Initial *in vitro* transposition experiments in our laboratory have been performed, but maize HMG proteins did not stimulate *in vitro Ac* transposition (Ruth Adolphs, personal communication). It is worthwhile discussing whether perhaps the lack of replication function associated to the transposon in the *in vitro* system is the reason for this failure.

3.1.2.2 About other DNA replication specific factors and transposition

Rep geminivirus protein has been a powerful instrument in shedding light on biochemical pathways governing G1-S transition in plants in recent years. It has been shown to induce the expression of many components of the replication machinery in infected non proliferating host cells (Gutierrez, 1999; Gutierrez, 2000). The protoplasts used in this work can also be considered as non-proliferating cells. These components either upregulate some S-phase functions (thus creating a cellular environment that permits geminivirus DNA replication) or induce further host cell cycle progression. For example, the Tomato Golden Mosaic Virus (TGMV) replication protein AL1 is sufficient to induce the accumulation of a factor functionally equivalent to animal PCNA (proliferating cell nuclear antigen) in terminally differentiated cells of transgenic tobacco plants (Nagar *et al*., 1995). In the absence of Rep function, the concentration of this factor becomes significantly decreased. PCNA plays an essential role in both replicative and repair DNA synthesis by interaction with cell-cycle regulatory proteins and functioning as a DNA

sliding clamp for the DNA polymerase delta. Only actively dividing plant cells contain high levels of this protein. A PCNA binding motif has been identified in the transposases encoded by *Pogo* (Drosophila) and *Tigger* (human) DNA transposons (Warbrick *et al.*, 1998), belonging to the *Tc1/Mariner* superfamily of transposons. This raises interesting possibilities for a novel mechanism of transposition in which the transposase might be targeted to replicating DNA. Unfortunately, no functional studies have been undertaken on these elements since *Pogo* and *Tigger* appear to have lost the capability to transpose. It would be interesting to look for similar interactions in *Ac* transposase.

3.1.3 DNA replication and effects on footprint formation

The excision of *Ac* and *Ds* elements is associated with the formation of characteristic transposon 'footprints' at the empty donor site that are the products of DNA end joining and host repair reactions. Several general trends have been observed among the footprints reported to date. Each excision event leaves its own footprint, and, for any given insertion, the footprint varies in sequence and number of bases from the original target site duplication. Most probably, sequences surrounding the element represent an important constraint on footprint formation. Bases near the center of each footprint often become replaced by their complements. Finally, footprints with fewer bases deleted from the original target site duplication are more prevalent than those that have lost more bases.

By analysis of the footprints left by plant transposons on their donor site, two models have been developed to explain the transposition mechanism: the "exonuclease" model (Saedler and Nevers, 1985) and the "endonuclease" model (Coen *et al.*, 1989), both recently modified by Scott and coworkers (Scott *et al.*, 1996) and Rinehart and coworkers (Rinehart *et al.*, 1997). Central to both models, and in general all transposition reactions, are breakage events that precisely expose the 3' ends of the transposable element. These exposed 3' ends are then joined to the target DNA.

In the "exonuclease" model (Fig. 23A), transposase creates staggered nicks precisely at the ends of the target site duplications. Subsequently, a 5' to 3' exonuclease removes nucleotides from the single strand overhangs protruding from the flanking DNA while DNA synthesis occurs at the available 3' ends until the element is removed. The empty site is rejoined, and mismatched or unmatched bases are corrected. Bases replaced by their complements and short inversions arise in the middle of footprints when DNA polymerase

switches strands to use the single-stranded overhangs as template. The exonuclease has poor processivity and it removes the 5' flanking sequence base adjacent to the element much more readily than any subsequent bases.

According to the "endonuclease" (also named "hairpin-intermediate") model (Fig. 23B), initially proposed based on transposon footprints left by *Tam* elements in *Anthirrinum majus*, single-strand cuts staggered by only one residue are introduced at the transposon ends, producing flanking DNA ends each having a single nucleotide overhang. The element is then removed and the remaining DNA ends close on themselves to form hairpin structures. An endonuclease (transposase itself?) then cleaves each hairpin at a random site, the opened ends are ligated to each other and mismatched or unmatched bases are corrected. Exonuclease degradation could occur after cleavage of the hairpin loops but before the opened loops are religated. This accounts for the small deletions often observed on the target duplication sequences.

The influence of DNA replication on transposition excision mechanism has been investigated in this work. Footprints left behind after *Ds* excision on a replicating and non-replicating donor site have been compared. Such a comparative analysis has never been performed before.

3.1.3.1 General footprint-characterization

Most of the footprints, whether in replication or non-replication context, are consistent with the deletion or replacement by its complement of the base(s) immediately flanking the *Ds*. Deletions rarely extend beyond the target site duplication and in a few cases they are associated with DNA insertions. Moreover, no remainings of the transposon ends are observed, indicating the complete removal of the transposon sequence during excision. A non-random end-joining repair occurs after *Ds* excision, as evidenced by the identification of two predominant excision products, '*gc*' and '*g*', of which the more frequent one, '*gc*', is characterized by transversion of the nucleotides immediately flanking the *Ds* on both sides. Accordingly, these data show close similarity with *Ac*/*Ds* footprints observed after excision from maize and transgenic Arabidopsis genomes (Scott *et al.*, 1996; Rinehart *et al.*, 1997) and from other geminivirus reporter vectors in monocot plants such as rice (Sugimoto *et al.*, 1994), wheat and maize (Laufs *et al.*, 1990), and barley (McElroy *et al.*, 1997) (where actually only footprints from replicating constructs could be analysed). In

these cases, footprints could be readily explained with both the "exonuclease" and the "hairpin-intermediate" models. Figure 23 shows two models for the formation of the predominant footprints observed in the present work by either the "exonuclease" or the "endonuclease" pathway.

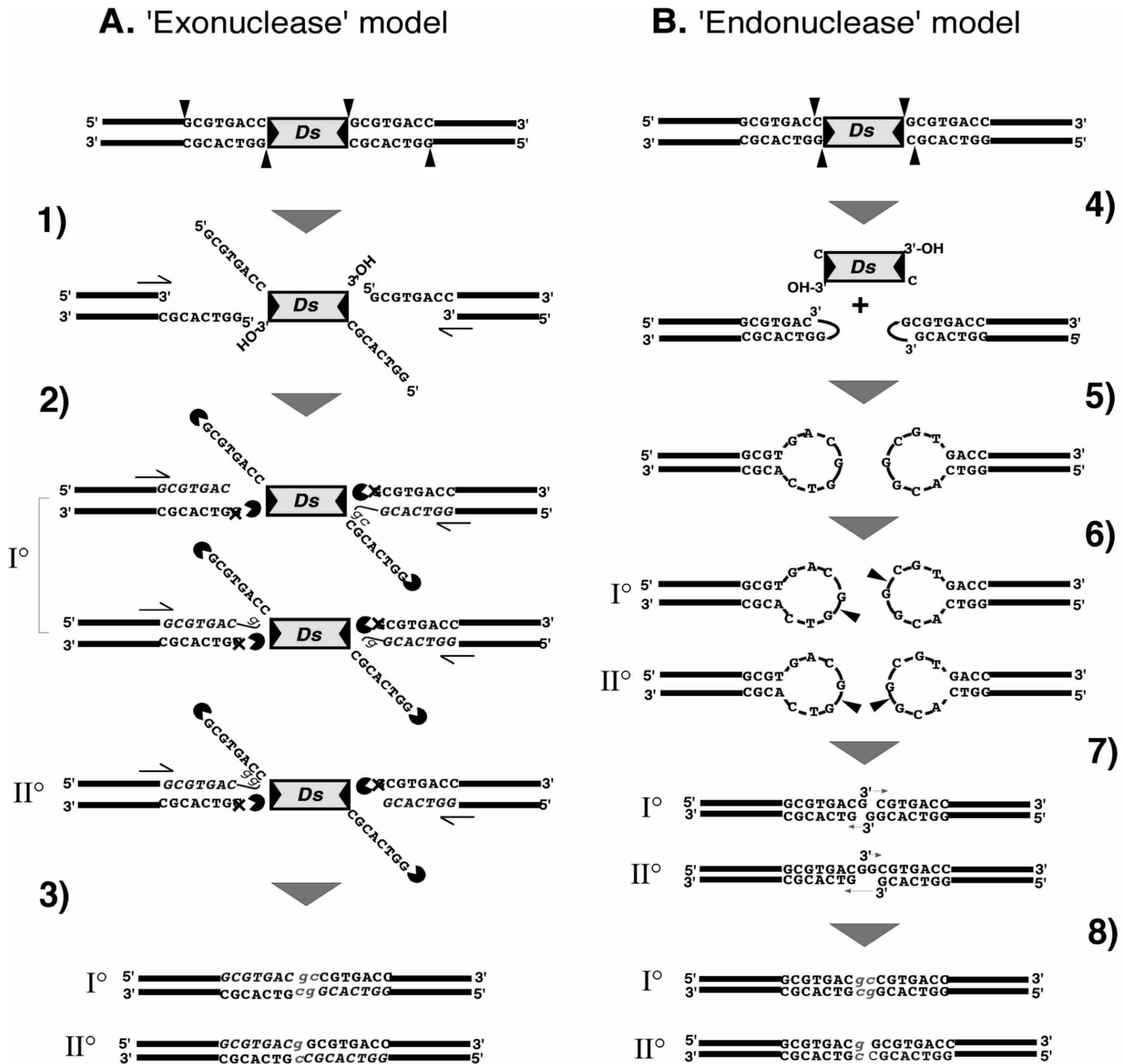


Fig. 23. "Exonuclease" and "Endonuclease" transposable element excision models. The two predominant *Ds* footprints observed in the present work can be explained by both models. I°: footprint '*gc*'; II°: footprint '*g*'. As proposed by the "exonuclease" model: **1)** Transposase introduces 8 bp staggered cuts at each end of the element; the element persists at the site of excision. **2)** DNA synthesis occurs at the

free 3' ends, while a 5' to 3' exonuclease begins to degradate the 5' overhangs on excised *Ds* element and bases adjacent to the element; the predominant footprint '*gc*' can originate by DNA polymerase switching on both or only the right 5' overhang, whereas DNA polymerase switching on the left 5' overhang could account for the footprint '*g*'. **3)** Once the element is removed, the DNA ends of the empty site are religated and any mismatched base pairs are corrected. As proposed by the "endonuclease" model: **4)** Transposase introduces 1 bp staggered cuts at each end of the element; the element is removed immediately after transposase cleavage and the remaining donor site ends close on themselves through the exposed 3' OH ends. **(5)** Hairpin structures are formed. **(6)** Endonucleolytic cleavage occurs on both hairpins; arrowheads indicate the positions of the cuts accounting for footprint '*gc*' and '*g*' formation respectively. **(7)** Hairpin resolved ends are ligated to each other. **(8)** Mismatched or unmatched base pairs are corrected.

To this end it is still unclear which of the two models proposed for footprint formation is responsible for the characteristic *Ac*/*Ds* excision products generated in plants.
It was recently demonstrated that *Ac*/*Ds* elements can transpose in yeast (Weil and Kunze, 2000). In contrast to *Ac*/*Ds* behaviour in plants, in yeast all excision footprints show traces of both ends of *Ac*/*Ds* elements. Moreover, they are characterized by the presence of palindromic duplications of the flanking host sequence centered around the 5'-terminal base of the *Ds* element. In this case, these excision products are more compatible with a "hairpin-intermediate" model (Coen *et al.*, 1989). In plants, these excision products are rare for *Ac*/*Ds* (0.1%) (Rinehart *et al.*, 1997). In the present work palindromic duplications do not appear at all. However, indirect support for the hairpin mechanism was obtained by the study of extrachromosomal *Ac*/*Ds* elements (Gorbunova and Levy, 2000). As it appears unlikely that *Ac*/*Ds* elements excise with alternate mechanisms in different environments, it is conceivable that the *Ac*/*Ds* excision reaction follows the hairpin-intermediate pathway; probably factors in maize might strongly influence that mechanism in ways not observed in other systems.
The "hairpin-intermediate" model was also recently used to explain RAG1/RAG2 mediated immunoglobulin gene rearrangement in V(D)J recombination, where direct evidence for hairpin structures is described (Roth *et al.*, 1992; van Gent *et al.*, 1995; Agrawal *et al.*, 1998; Hiom *et al.*, 1998). Also, other members of the "hAt" superfamily such as *hobo* (Atkinson *et al.*, 1993), *Tam3* (Coen *et al.*, 1989) and *Ascot* (Colot *et al.*, 1998) appear to follow the same mechanism.

3.1.3.2 Comparing footprints left by a replicating and a non-replicating *Ds* element

a) Replication does not influence the basic DNA repair mechanism and enzyme pathway involved during footprint formation since the predominant footprint is the same on replicating and non-replicating reporter plasmids.

Instead, replication appears to enhance the fidelity of the reactions leading to the formation of the predominant footprint, as suggested by the almost five fold higher frequency of footprints deviating from the dominant *'gc'* observed in replication deficient donor sites. Since replication generally increases the capability of *Ac/Ds* elements to transpose, it is conceivable that such a parallel increase in the fidelity of the repair processes has been developed to preserve the host genome from an otherwise deleterious mutation rate. Further implications are discussed below.

b) It is interesting to observe that the processing of the two flanks at the *Ds* excision site exhibits a certain bias at the non-predominant footprints in the "non-replication context" (Fig. 16B). At the right flank only limited deletions are formed that do not extend beyond the target site duplications, whereas at the left flank more than half of the non-predominant footprints exhibit more extensive deletions.

No bias in excision site formation can be recognized in the "replication context" (Fig. 16A), but here the small number of non-predominant footprints does not allow a statistically significant statement.

Such different behaviour in the processing of the two flanks at the *Ds* excision site was seen also by *Ds*-like transposon *Ascot-1* excision footprints in *Ascobulus immersus* (Colot *et al.*, 1998), where an hairpin intermediate was proposed to explain footprint formation. Also in this case a wider spectrum of deletions occurs at the left flank of the transposon donor site. Non-random positioning of the single-strand nick required for hairpin resolution has been recently demonstrated for V(D)J recombination (Nadel and Feeney, 1995; Gauss and Lieber, 1996; Ezekiel *et al.*, 1997; Nadel and Feeney, 1997). These studies have led to the conclusion that hairpin sequences play a determinant role in dictating where resolution takes place.

3.1.3.3 How could DNA replication promote DNA repair reactions?

Transposon footprints are the product of transposase-mediated cutting, followed by DNA end-joining repair either by the transposase itself, by host repair functions or a combination of both.

The occurrence of a predominant footprint in *Ac/Ds* excision suggests a tightly controlled reaction pathway regulating the repair of the damaged site. Less frequent, related footprints can be interpreted as products of interrupted or disturbed repair processes.

Little is known about the plant genes involved in DNA repair; whether they interact with the transposition complex (transpososome), how they are regulated and what interferes with their activity. Data reported here indicate that factors "disturbing" repair process are less efficient if the excision site is repaired inside a replication fork, so that the entire repair mechanism becomes more accurate. How could DNA replication influence the repair processes?

3.1.3.3.1 Influence of DNA topology changes during replication

The "endonuclease" model, as modified by Scott *et al.* (1996), proposes an exonuclease degradation occurring between hairpin cleavage and religation of opened loops in addition to the hairpin-resolving endonuclease cutting. It is conceivable that either DNA conformational changes or specific components involved in these changes, such as DNA bending proteins around the replication fork, contribute to a more specific cleavage inside the hairpin, and serves to retain the resolved hairpin ends closer to each other. This would make the endonuclease-cutting/end-rejoining reaction occur in a more rapid succession, so that possible exonucleases present in the proximity do not have much time to act. This closely resembles the action of the IRBP factor in Drosophila *P* element transposition. Supposedly, IRBP binds to free DNA ends and protects them from exonuclease degradation or may promote the joining of double-strand DNA breaks by modulating the activity of other repair proteins (Beall and Rio, 1996).
Such topological DNA influences of replication in footprint formation can also be proposed for the "exonuclease" model. On the one hand DNA topology might affect the efficiency of the rejoining/repair reaction; on the other hand, DNA overhangs (supposedly sequestered by DNA bending proteins or by a even more stable TPase/DNA complex) might be less susceptible to degradation by a host-encoded exonuclease.

3.1.3.3.2 Influences of enzymology during DNA synthesis

Together with DNA topology, the enzymology of the DNA repair apparatus might be influenced by the proximity of a replication fork.
DNA replication and DNA repair are two very closely related processes in the cell. Several mechanisms are involved to ensure the fidelity of replication, including a balanced level of dNTPs provided by the fine regulation of key biosynthetic steps, and 'induced fits' of

polymerase and DNA. Also important is the proofreading activity of polymerases by 3'-5' exonuclease, such as polymerases δ and ε. The above-mentioned PCNA factor, whose production in the host cell is found to be induced by the Rep geminivirus protein (Nagar *et al*., 1995), builds the moving platform for DNA polymerase δ together with RF-C factor (Umar *et al*., 1996). This platform is required for the efficient repair of mismatched DNA (Longley *et al*., 1997). Acting fundamentally as an important DNA bending factor, PCNA signals the presence of DNA lesions to the DNA damage checkpoint machinery (Tsurimoto, 1999; Moggs *et al*., 2000).
It is conceivable that such a scenario by the proximity of the replication machinery positively influences also the fidelity of the repair processes at a replicating transposon donor site.

3.2 Transposition regulation by DNA methylation in conjunction with replication

Pioneering observations on the epigenetic regulation of maize transposons were reported by Barbara McClintock's studies in the fifties. McClintock observed that maize transposons could change to a number of different forms in which they either are silent, or they alternate between active and inactive phases during development (McClintock, 1957; McClintock, 1958; McClintock, 1959; McClintock, 1965). She described different developmental patterns of transposon activity and reported that a given developmental pattern is heritable, yet subject to further heritable changes. She also found that elements could remain silent for multiple plant generations, returning to an active form at a very low frequency. She made the seminal observation that an inactive element could be activated by the presence of an active element, suggesting the existence of a transposon-encoded epigenetic activator (McClintock, 1971). McClintock concluded that transposable elements are maintained in genomes in a deeply inactive, yet genetically responsive state, now designated 'cryptic' state. In support of this inference, she showed that previously undetectable transposable elements could be converted to an active form under circumstances of extensive chromosome breakage, as a response (she interpreted) of the genome to adapt when confronted with challenging conditions (McClintock, 1951). These observations revealed the existence of a reversible (and therefore epigenetic) inactivation mechanism. Now we know that this inactivation mechanism is methylation-associated and

likely has evolved by the host cell to control the potentially deleterious effects of transposition.

Two main roles of DNA C-methylation have been described on the regulation of *Ac*/*Ds* transposition. C-methylation can transcriptionally and reversibly inactivate or modulate the *Ac* promoter. In the presence of a C-methylated promoter the *Ac* TPase expression is dramatically reduced to 2% or less, and the trace amounts of TPase protein expressed from the residual *Ac* mRNA are not sufficient to induce transposition events at a detectable level (Kunze *et al.*, 1988; Brutnell and Dellaporta, 1994). The inactivated element can be reactivated by the presence of a second active element in the genome over several generations (Brutnell and Dellaporta, 1994). Changes of phase associated with C-methylation and loss of activity have been described also for other maize transposons such as *En*/*Spm* (Banks and Fedoroff, 1989) and *Mutator* (Chandler and Walbot, 1986; Bennetzen, 1987); some indications are reported for the *Antirrhinum majus Tam 3* element (Martin *et al.*, 1989).

In addition to TPase gene silencing, a second regulatory function of DNA C-methylation has been proposed. It relies on modified transposase binding sites and accounts for the coupling of transposition to replication, providing the differential transpositional competence of *Ac*/*Ds* before replication and respectively of the two daughter elements after replication. In other words it accounts for the model of chromatid selectivity proposed by Wang and coworkers (Wang *et al.*, 1996; Wang and Kunze, 1998).

3.2.1 Before replication, DNA holomethylation prevents element excision

An important prerequisite for the validity of the model of Wang and colleagues is that, before replication (therefore in general during cell cycle phases other than replication), TPase binding sites are hypermethylated and the transposon supposedly inactive. Either no transposase is present because the *Ac* promoter which overlaps with the TPase binding sites is silenced, or in the presence of transposase, the transposon is in a state which presumably is not recognized by the transposition machinery, as suggested by the very low affinity of the transposase to holomethylated synthetic binding sites in gel retardation assays (Kunze and Starlinger, 1989). Also in *En*/*Spm*, a maize transposon of the so called CACTA family, methylation at both ends of the element renders the *En*/*Spm* promoter inactive. Moreover, methylated *dSpm* elements are, as well, reduced in excision frequency

when transactivated by an active *En/Spm* element (Banks and Fedoroff, 1989). Methylated ends appear to inhibit the excision of the element, probably because TNPA binding is hampered (Gierl *et al*., 1988).

In transgenic plants the effect of TPase binding site methylation on transposition can not directly be studied, since it is not possible to manipulate the methylation state. Therefore, in this work a procedure was developed to examine the consequences of TPase binding site methylation in a transient transposition system in transfected petunia protoplasts.
Fortunately, the methylation pattern of the inserted extrachromosomal DNA was stably mantained in the petunia cells after transfection during culturing time (Fig. 14). The efficiency of the *Ac* TPase to excise *Ds* decreases of factor 12 in the absence of replication and in the presence of holomethylated binding sites (Fig. 19). A reason for the residual transposition activity observed could be that the *in vitro* methylated plasmid is very weakly demethylated once it is transfected into the cell, at a level that is not detectable by Southern analysis, but sufficient to allow a very low activity of the transposase. Alternatively, the binding of the TPase to holomethylated ends is weak but enough to account for a low functionality of the enzyme. One can also imagine that the TPase induces demethylation of its own binding sites at a very low rate. It is known that the presence of an active transposon in the genome can accelerate the re-activation process of a silenced (hypermethylated) one (McClintock, 1971; Schwartz and Dennis, 1986; Kunze *et al*., 1988). The most plausible explanation is that postreplicative TPase binding to the (then hemimethylated) silenced transposon facilitates the simultaneous binding of transcription factors to the promoter preventing the maintenance methylation of the element. Although unlikely, one can not completely rule out that transposase itself overcomes the methylation impediment through an intrinsic demethylase activity.

3.2.1.1 By what mechanism blocks DNA methylation transposase action?

DNA methylation prevents the binding of the transposase itself to its binding sites. This would be the most direct mechanism by which DNA methylation blocks transposase action. This appears a plausible scenario, since in gel retardation assays weak or no TPase binding is observed on holomethylated synthetic target site oligos (Kunze and Starlinger, 1989) and complete *Ds* 5' and 3' subterminal regions (this work).

Nevertheless, more indirect mechanisms might also be involved. Studies with HeLa cells have shown that the reduction in affinity of transcription factors for their own methylated promoters is often insufficient to account for the inactivity of promoters *in vivo* (Iguchi-Ariga and Schaffner, 1989; Weih *et al*., 1991). Moreover, the MURA transposase of maize *Mutator* element, also epigenetically regulated by DNA C-methylation, is capable of binding a holomethylated substrate, although holomethylated elements are not transposed *in vivo* (Benito and Walbot, 1997).

In vertebrates, two methyl-CpG binding proteins have been identified and well characterized, MeCP1 and MeCP2. These repress DNA transcription factors activity, by binding to methylated promoters without apparent sequence specificity (Lewis *et al*., 1992; Meehan *et al*., 1992) and in association with histone deacetylases (Jones *et al*., 1998; Nan *et al*., 1998). Interestingly, MeCP2 contains a methyl-CpG DNA-binding domain, which might alter chromatin structure directly, and a repressor domain, which might function indirectly to confer long-range repression *in vivo* (Nan *et al*., 1993; Nan *et al*., 1997). In plant, the Arabidopsis MOM gene represents the first candidate for such methylation-sensing factors. This gene was recently isolated by Amedeo and colleagues (Amedeo *et al*., 2000) and is required for the maintenance of transcriptional gene silencing. Mutation of this gene reactivates transcription from several previously silent, heavily methylated loci. Despite this, the dense methylation at these reactivated loci is maintained, indicating that MOM may act downstream of methylation in epigenetic regulation. It is conceivable that similar specific modulators that recognize methyl-CpG prevent the binding also of the TPase to the transposon ends, either occupying TPase binding sites or modifying chromatin structure in a way that TPase can not approach the target DNA.

3.2.1.2 *De novo* methylation

Normally, *in vivo*, the methylated nucleotides are largely restricted to the transposable element so that the transposon behaves like a CpG methylation island. It is interesting to contemplate how *Ac* can be specifically recognized and targeted by the host methylation machinery for *de novo* C-methylation.

- Is hypermethylation the cause or the consequence of the inactivation process?

The question as to whether local hypermethylation is cause or consequence of the inactivation process is still open. It has been shown in transgenic tobacco lines that

cytosine methylation at CpG and CpNpG sequences is not a prerequisite for the initiation of transcriptional gene silencing, as it occurs before any cytosine residue is methylated. It has been suggested that the silencing would then recruit methyltransferase to the affected region as a requirement for the maintenance of the silent state (Dieguez *et al.*, 1998).
In contrast, in *ddm1 Arabidopsis thaliana* DNA methylation mutants (Vongs *et al.*, 1993), where genomic cytosine methylation is reduced to 25 to 30% of the amount observed in wild-type plants, gene silencing stopped in the absence of methylation (Jeddeloh *et al.*, 1998). Either *ddm1* mutations suppress gene silencing directly through a reduction in DNA methylation of the silenced loci, or they affect chromatin structure with consequent DNA hypomethylation and loss of gene silencing.

• How are transposable elements specifically targeted?
In *Spm*, signals for the *de novo* methylation of the element are included in the element itself (Schläppi *et al.*, 1994). The downstream untranslated control region (DCR) of the element, consisting of a 350 bp GC-rich region is required for *de novo* methylation and appears to initally attract the cellular methylation machinery. In *Ac* there is no clear data regarding whether its untranslated leader sequence has a particular function, but it is known that its methylation is accompanied by a dramatically reduced transcript level (Kunze *et al.*, 1988). As during transition from the active to the inactive state the 5' end of *Ac* gets *de novo* methylated (Schwartz and Dennis, 1986; Kunze *et al.*, 1987), it is conceivable that the element contains signals that specifically direct the methylation machinery to its 5' end.
At least two ways are proposed regarding how transposable elements might be recognized as invasive and acted upon by the DNA methylation machinery (Bestor and Tycko, 1996). Integration intermediates, which form when transposable elements insert into a genome, can provide targets for *de novo* methylation. Or, a second likely signal is pairing of transposon multiple copies at ectopic locations: unique features of the paired regions mark them for methylation along the lengths of the interacting repeats. Such a mechanism implies that one type of cytosine-*de novo* methyltransferase activity in eukaryotes is devoted to surveillance for aberrant paired DNA species. According to this model, efficiency of *de novo* methylation is increased when repeats are linked to each other in an inverted repeat arrangement, probably because of the increased stability of pairing in this structure. Such a mechanism is invoked for example to explain methylation and silencing

of duplicated sequences (Selker and Stevens, 1987) and the related mechanism of the Repeat-Induced Point mutation (RIP) (Selker *et al*., 1993; Perkins *et al*., 1997) in *Neurospora crassa*. In plants, a related mechanism in *Arabidopsis thaliana* is involved in the *de novo* methylation of complex concatemeric arrays of two or more transgene copies inserted into a single locus (Assaad *et al*., 1993; Davies *et al*., 1997).

3.2.2 C-hemimethylation accounts for different transpositional competence of the two daughter elements after replication

Once *Ac* or *Ds* are methylated, their transposition is prevented. Once the DNA replicates, two transiently hemimethylated daughter elements are formed. In this context, as postulated by the model of Wang and coworkers, the transposon regains its competence for transposition, at least at the level of transposase recognition sites: efficient binding capacity of the transposase to its binding sites should in fact be restored. Moreover, according to the model, only one of the two hemimethylated sister elements should contribute to the restoration of the transposition competence.

3.2.2.1 Identification of the chromatide competent for transposition

The results show that a methylated transposon regains its transposition competence following replication (Results section 2.3.1.3). By constructing non-replicating *Ds* reporter plasmids where either the top or the bottom strand of the *Ds* element was hemimethylated, it was demonstrated that the transposase is active on postreplicative hemimethylated *Ds* and preferentially excises the *Ds* element that is C-methylated at the top strand.

It remains unclear whether the 6.3-fold difference in transposition activity between the two daughter elements in the artificial petunia system reflects the situation of an element in the maize genome. A maize transposable element is in fact studied in an heterologous plant system; moreover, the plasmid vectors supposedly differ in topology and association with proteins from the chromosomal chromatin structure. Also, in these experiments every cytosine and CpG groups are modified to the same extent in the synthetically methylated *Ds* elements, whereas in maize C-methylation involves preferential sites in the epigenetically inactivated *Ds-cy* element (Wang and Kunze, 1998). The uneven distribution of ^{5m}C residues is all the more emphasized in the active *Ac9* element (Wang and Kunze, 1996) (see Introduction section 1.3.4.2).

The data are consistent with the proposed model of chromatid selectivity. Methylation status of *cis*-acting sequences is one of the possible mechanisms for *Ac* transposition coupling to DNA replication.

This cell cycle-dependence of transposition in conjunction with DNA C-methylation has been described to date only in *Ac/Ds* among all plant transposons. *En/Spm* transposition is connected to DNA replication since the same types of twin sectors are observed with *En/Spm* as with *Ac* (Dash and Peterson, 1994); however, this connection is possibly through something other than hemimethylation if mediated by TNPA transposase. Binding of TNPA protein to hemimethylated forms of the binding motif (also containing a CpG sequence) is reduced by a factor 5 to 10 compared to the non methylated form and both hemimethylated forms are equally recognized by the transposase (Gierl *et al*., 1988). In the maize *Mutato*r transposable element MURA transposase binds preferentially one hemimethylated form of the 32 bp MURA Binding Site (MBS) than the other. However strand selectivity and coupling of transposition with replication are not yet demonstrated for this maize transposon (Benito and Walbot, 1997).

3.2.2.2 The transposition competence of the hemimethylated *Ds* active state correlates with the transposase occupation of its 5' and 3' end

The *in vitro* transposase binding studies that contributed to the formulation of the model for chromatid selectivity were performed on synthetic, differentially methylated concatamers of the binding sites (Kunze and Starlinger, 1989). These sites do not resemble the transposon ends. In order to complete the analysis of *Ac* chromatid selectivity determinants, a detailed characterization of TPase binding affinities to its binding motifs in their native sequence environment, at both 5' and 3' ends of the competent and incompetent *Ds* elements, has been performed. In the active state, both *Ds* ends are efficiently complexed by the *Ac* transposase (Results section 2.3.2.3). In the inactive state, only the small central group of six binding sites of the 5' end shows high affinity for the transposase, whereas no transposase binding is observed at the motifs bordering, at both ends, the terminal inverted repeat.

In previous analyses (on unmethylated *Ds*), it has been shown how mutations in the TIRs that abolish TPase binding result in the total immobilization of the element (Hehl and Baker, 1989; Healy *et al*., 1993). Moreover, progressive deletion of subterminal TPase binding sites from the center of *Ac/Ds* toward its borders results in a progressive reduction

in transposition frequency (Coupland *et al*., 1989). A mutation in the single 3'-subterminal TPase binding site adjacent to the TIR almost completely inhibits transposition (Weil and Kunze, 2000). Similarly, at the 5' end point mutations in the three TPase binding sites adjacent to the 5' TIR reduce *Ds* excision to approximately 6% of the wild type level (Chatterjee and Starlinger, 1995). In summary these results can be interpreted to indicate that the most distal subterminal TPase binding sites (those immediately bordering the TIRs) are essential for transposition; these motifs are those that are specifically complexed by the transposase in the identified competent *Ds* state. We can therefore conclude that the two alternative hemimethylation states are *cis*-acting determinants of the transposition activity of the *Ds* element.

3.3 Perspectives

Ds transposition is enhanced by DNA replication independently of the methylation status of the element and in dicots replication-dependence is less pronounced than in monocots.

It would be interesting to understand which components of the DNA replication machinery are affecting *Ac* transposition at the molecular level and what differentiates monocot and dicot hosts in the way this influence occurs.
The use of yeast as host system for *Ac*/*Ds* transposition analyses could be very helpful. Recent studies showed that a mini*Ds* element placed in the cells of the budding yeast *Saccharomyces cerevisiae* can transpose when *Ac* TPase is provided to it *in trans* (Weil and Kunze, 2000). The large amount of yeast mutants at cell cycle specific components and the vast information already available on G1-S transition in yeast, could represent a powerful workshop for basic and rapid investigations on specific replication factors interacting with *Ac* transposition complex and modulating its activity.

4 MATERIAL AND METHODS

4.1 MATERIAL

4.1.1 Chemicals

Chemicals employed in this work, were purchased from the companies Amersham-Pharmacia, BIO101, Biozym, Difco, Merck, Duchefa, Boehringer-Roche, Roth, Serva and Sigma-Aldrich-Fluka. All chemicals used for the petunia protoplast transient assay were purchased from the companies Sigma-Aldrich, Duchefa and BIOSYNTH. Radiochemicals were purchased from NEN™ Life Science. dNTPs were furnished by PEQLAB; ^{5m}dCTP and ATP were furnished by Amersham-Pharmacia.

4.1.2 Enzymes

Tab. 5. Enzymes employed in this work

Enzyme	Supplier Company
AmpliTaq-Polymerase	ABI
Calf Intestine Alkaline Phosphatase	MBI Fermentas
Cellulase "onozuka R-10" (*Trichoderma v.*)	SERVA
DNase I-RNase free	Boehringer-Roche
Lysozyme	Merck
Macerozyme R-10 (*Rhizopus sp. lyophil.*)	SERVA
Pfu DNA Polymerase	Stratagene
Restriction endonucleases	Gibco BRL, MBI Fermentas, Amersham-Pharmacia, New England Biolabs (NEB) and Boehringer-Roche
Shrimp Alkaline Phosphatase	Amersham-Pharmacia
Sss I-CpG Methylase	New England Biolabs (NEB)
T4 DNA Ligase	Boehringer-Roche
T4 DNA Polymerase	New England Biolabs (NEB)
T4 Polynucleotide Kinase	MBI Fermentas
Taq DNA Polymerase	AGS GmbH

4.1.3 Kits

TOPO TA Cloning® (Invitrogen) for cloning of PCR products, DNeasy Plant Mini Kit (Qiagen) for extraction of genomic DNA from protoplasts, Plasmid Maxi Kit (Qiagen) for plasmid maxi preparations, GFX™ PCR and Gel Band Purification Kit (Amersham-

Pharmacia) for purification of amplified DNA, Micro Spin™ S-300 HR, S-400 HR and G-25 columns (Amersham-Pharmacia) for gel filtration DNA purification, Ready To Go™ DNA Labelling Beads (-dCTP/-dATP) (Amersham-Pharmacia) for radiactive ^{32}P-labeling of DNA probes, BigDye Terminator Cycle Sequencing Ready Reaction (ABI) for DNA sequencing.

4.1.4 *Escherichia coli* strains

Tab. 6. *E. coli* strains employed in this work

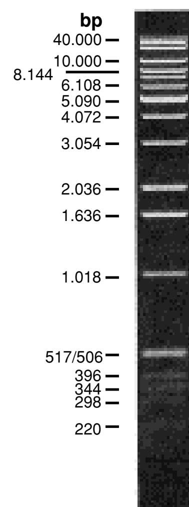
Strain	Genotype	Use
DH5α	F', *endA1, hsdR, hsdM, sup44, thi-1, gyrA1, gyrA96, relA1, recA1, lacZΔM15*	Cloning, propagation and isolation of *dam-dcm*-methylated plasmids.
JM110	*dam dcm supE*44 *hsdR*17 *thi-1 leu rpsL* (Str^r) l*acY galK galT ara tonA thr tsx* Δ(*lac-proAB*) [F' *traD36 proAB lacI*^{q}*lac*ZΔM15]	Propagation and isolation of *dam-dcm*-unmethylated plasmids.
NM522	*supE*, *thi-1*, Δ(*hsdSM-mcrB*)5, Δ(*lac-proAB*), [F' *proAB lacI*^{q}*lacZΔM15*]	Preparation of phagemid single stranded DNA.

4.1.5 Plant material

Seed material of *Petunia hybrida* [ssp. RL01 × ssp. Blue Titanic] was kindly provided by Iris Heidemann (Max-Planck-Institut für Züchtungsforschung, Cologne, Germany).

4.1.6 DNA-length molecular standard

1 kbp-DNA-Extension-Ladder from GibcoBRL™ was used as DNA-length standard.



4.1.7 Synthetic oligonucleotides

All oligonucleotides were purchased from Amersham-Pharmacia or Metabion. The following nucleotides were used.

Tab. 7. Oligonucleotides used for DNA amplification and sequencing

Name	Sequence	Use
bDs2	Biot-GACCCAGGGATGAAAGTAGGATGGGA AAATCCC	Amplification of the *Ds* element from *Ds* reporter plasmid. It hybridizes with the *Ds* 5' end.
bDs3	Biot-CGGTCGGTAACGGTCGGTAAAATACCT CTA	Amplification of the *Ds* element from *Ds* reporter plasmid. It hybridizes with the *Ds* 3' end.
Ds2.II	GACCCAGGGATGAAAGTAGGATGGGAAAAT CC	Amplification of the *Ds* element from *Ds* reporter plasmid. It hybridizes with the *Ds* 5' end.
Ds3	CGGTCGGTAACGGTCGGTAAAATACCTCTA	Amplification of the *Ds* element from *Ds* reporter plasmid. It hybridizes with the *Ds* 3' end.
mDs3	^{5m}CGGT^{5m}CGGTAA^{5m}CGGT^{5m}CGGTAAAATA^{5m}C ^{5m}CT^{5m}CTA	Amplification of the *Ds* element from *Ds* reporter plasmid. It hybridizes with the *Ds* 3' end.
OLIGO 1	TCCCGTACCGACCGTTATCGTATAACCGATT TTGTTAGTTTTATCCCGATCGATTTCGAACCC GAGG	Gel retardation assay with the *Ds* 5' end sequence: nu. 30-96.
OLIGO 1 MET	TC^{5m}C^{5m}CGTA^{5m}C^{5m}CGA^{5m}C^{5m}CGTTATCGTATA A^{5m}C^{5m}CGATTTTGTTAGTTTTATC^{5m}C^{5m}CGATC GATTTCGAAC^{5m} C^{5m}CGAGG	Gel retardation assay with the *Ds* 5' end sequence: nu. 30-96.
OLIGO 2	CCTCGGGTTCGAAATCGATCGGGATAAAACT AACAAAATCGGTTATACGATAACGGTCGGT ACGGGA	Gel retardation assay with the *Ds* 5' end sequence: nu. 96-30.
OLIGO 2 MET	CCT^{5m}CGGGTTCGAAATCGAT^{5m}CGGGATAAA ACTAACAAAAT^{5m}CGGTTATACGATAA^{5m}CGG T^{5m}CGGTA^{5m}CGGGA	Gel retardation assay with the *Ds* 5' end sequence: nu. 96-30.
OLIGO 3	AAAACGGAACGGAAACGGGATATACAAAAC GGTAAACGGAAACGGAAACGGTA	Gel retardation assay with the *Ds* 5' end sequence: nu. 106-158.
OLIGO 3 MET	AAAA^{5m}CGGAA^{5m}CGGAAA^{5m}CGGGATATACA AAA^{5m}CGGTAAA^{5m}CGGAAA^{5m}CGGAAA^{5m}CGG TA	Gel retardation assay with the *Ds* 5' end sequence: nu. 106-158.
OLIGO 4 MET	TA^{5m}C^{5m}CGTTT^{5m}C^{5m}CGTTT^{5m}C^{5m}CGTTTA^{5m}C^{5m} CGTTTTGTATATC^{5m}C^{5m}CGTTT^{5m}C^{5m}CGTT^{5m}C^{5m} CGTTTT	Gel retardation assay with the *Ds* 5' end sequence: nu. 158-106.

Name	Sequence	Use
OLIGO 5	TCCCGACCGTTTCACCGGGATCCCGTTTTTA ATCGGGATGATCCCGTTTCGTTACCGTATTT TCTAATTCGGGAT	Gel retardation assay with the *Ds* 5' end sequence: nu. 168-242.
OLIGO 5 MET	TC^{5m}C^{5m}CGA^{5m}C^{5m}CGTTTCA^{5m}C^{5m}CGGGATC^{5m}C ^{5m}CGTTTTTAAT^{5m}CGGGATGATC^{5m}C^{5m}CGTTTC GTTA^{5m}C^{5m}CGTATTTTCTAATT^{5m}CGGGAT	Gel retardation assay with the *Ds* 5' end sequence: nu. 168-242.
OLIGO 6	ATCCCGAATTAGAAAATACGGTAACGAAAC GGGATCATCCCGATTAAAAACGGGATCCCG GTGAAACGGTCGGGA	Gel retardation assay with the *Ds* 5' end sequence: nu. 242-168.
OLIGO 6 MET	ATC^{5m}C^{5m}CGAATTAGAAAATA^{5m}CGGTAACGA AA^{5m}CGGGATCATC^{5m}C^{5m}CGATTAAAAA^{5m}CGG GATCC^{5m}CGGTGAAA^{5m}CGGT^{5m}CGGGA	Gel retardation assay with the *Ds* 5' end sequence: nu. 242-168.
OLIGO4	TACCGTTTCCGTTTCCGTTTACCGTTTTGTAT ATCCCGTTTCCGTTCCGTTTT	Gel retardation assay with the *Ds* 5' end sequence: nu. 158-106.
opUC>350	AACGCCAGGGTTTTCCCAG	Sequencing of *Ds* excision footprints.
Pr.1	GGATACTTACGTCACGTCTTGCGCACTGAT	Amplification of *Ds* empty donor site from *Ds* reporter plasmids.
Pr.2	CCACAGTTTTCGCGATCCAGACTGAA	Amplification of *Ds* empty donor site from *Ds* reporter plasmids.

<u>Note</u>: Biot: biotinylated primers at the 5' end; ^{5m}C, 5-methyldeoxycytidine

4.1.8 Plasmids

Tab. 8. Plasmids employed in this work

Plasmid	Resistance	Reference	Description
pT7T3	Amp^R	Amersham-Pharmacia	Employed as source for the 414 bp *Ssp* I/*Bgl* I fragment corresponding to the f1 origin of replication of M13 bacteriophage.
pNT150	Amp^R	Becker, 1992 #20	Reporter plasmid derived from pNT150Ds (Becker *et al.*, 1992) by deletion of the *Ds* element as *Bam* HI fragment. The 1'-promoter (Velten *et al.*, 1984) controls the expression of the GUS-gene and the 2'-promoter (Velten *et al.*, 1984) that of the Luciferase gene.

Plasmid	Resistance	Reference	Description
pTYSst14	Amp^R	Kheyr-Pour, 1992 #19	It carries the 2770 bp genome of the monopartite geminivirus Tomato Yellow Leaf Curl Virus (TYLCV).
pNT600-10.ATG	Amp^R	Kunze, 1993 #21	Reporter plasmid that encodes for the $TPase_{103-807}$, a truncated version of the transposase protein under the control of the 1'-promoter (Velten *et al.*, 1984).

Tab. 9. Plasmids constructed in this work

Plasmid	Resistance	Description
pMiDsf1 and pMiDs<1f	Amp^R	Reporter plasmids derived from pNT150MiniDs (Becker *et al.*, 1992; Chatterjee and Starlinger, 1995), where a mini*Ds* element (Chatterjee and Starlinger, 1995), consisting of the terminal 246 bp of the *Ac* 5' end and 446 bp of the *Ac* 3' end, is inserted between the 1'-promoter and the GUS-gene. An f1-origin of replication of bacteriophage M13 was inserted in both orientations at the *Ehe* I site (nu. 235) between the GUS and the Amp (Ampicillin) resistance genes. f1(+): pMiDsf1, f1(–): pMiDs<1f.
pMiDsf1-RI	Amp^R	Reporter plasmid originated from pMiDsf1 by replacement of the *Kpn* I/*Ehe* I Luciferase gene fragment (1.8 kb) with the *cis*- (IR) and *trans*-acting (C1-C4) replication sequences of the TYLCV geminivirus genome (1.9 kb). Details of the cloning procedure are described in Results section 2.1.2.1.
pMiDsf1-rI	Amp^R	Reporter plasmid originated from pMiDsf1-RI by inversion of a 790 bp *Bst* 1107I/*Pfl* MI fragment internal to the C1 sequence.
pMiDsf1-R	Amp^R	Reporter plasmid originated from pMiDsf1-RI by deletion of a *Pfl* MI/*Nco* I 300 bp fragment corresponding to the IR sequence.
pMiDsf1-ΔRI	Amp^R	Reporter plasmid originated from pMiDsf1 by deletion of the *Kpn* I/*Ehe* I Luciferase gene fragment (1.8 kb).

4.1.9 Buffers and solutions

Tab. 10. Buffers and solutions employed in this work

Name	Recipe
Ampicillin solution	10% (w/v) Ampicillin in $H_20_{bid.}$, sterilfitered
Denhardt reagent (50×)	1% (w/v) bovine serum albumin (BSA) 1% (w/v) polyvinylpyrrolidon 1% (v/v) Ficoll
EMSA-binding buffer (5×)	75 mM Tris-HCl (pH 8) 300 mM NaCl 15 mM $MgCl_2$ 10 µM $ZnCl_2$ 50 mM DTT 0.25% (v/v) Triton-X-100 20% (v/v) Glycerin
EMSA-electrophoresis buffer	78 mM Tris-HCl (pH 8) 33 mM Na-acetate 10 mM EDTA
Fixation solution (GUS-staining)	10.9 mM MES 300 mM Mannit pH 5.6 with NaOH (810 µl 37% Formaldehyd per 100 ml solution are added immediately before use)
Hybridization solution (Southern)	6×SSPE 5×Denhardt reagent 0.1% (w/v) SDS 10 mM EDTA 100 µg/ml sonified denatured ST-DNA
K3 (petunia assay)	0.362% (w/v) Kao & Michayluk micro and macro elements (Duchefa) 3 mM $CaCl_2{\cdot}2H_2O$ 6 mM KNO_3 4.4 mM NH_4NO_3 1 mM $(NH_4)_2SO_4$ 0.8 mM $MgSO_4$ 0.1% (v/v) of steril filtered Vitamine-Stock solution (Duchefa, 8 mM Nicotinic acid 5 mM Pyridoxine-HCl 30 mM Thiamine-HCl) 1% (v/v) Ca-Phosphate stock solution (Fluka, 36.6mM$CaHPO_4{\cdot}2H_2O$, pH 3 with HCl, autoclaved) 0.6 mM myo-Inositol (Sigma) 1.7 mM D(+)-Xylose (Sigma) 0.4 M Sucrose (Duchefa) pH 5.6 with KOH 1M 0.001% (w/v) α-Naphthalene Acetic Acid (NAA, Sigma) 0.002% (w/v) Kinetine (Sigma) Osmolarity: 0.56±0.02 osmol, steril filtered

Name	Recipe
Loading buffer	0.1% Bromophenol blue 0.1% Xylen 50% Glycerol
Ma-Mg solution (petunia assay)	0.4 M Mannitol (Duchefa) 15 mM $MgCl_2 \cdot 6H_2O$ 0.1% (w/v) MES pH 5.6 with KOH, steril filtered
Na-Acetate buffer (pH 5.2)	3 M Na acetate pH 5.2 with glacial acetic acid
Na-Phosphate buffer (pH 7) (GUS-staining)	20 mM NaH_2PO_4 30 mM Na_2HPO_4
PEG solution (40%) (petunia assay)	4% (w/v) PEG_{6000} 0.1 M $Ca(NO_3)_2 \cdot H_2O$ 0.4 M Mannitol Heat to 70°C to dissolve PEG pH 9-9.4 with 0.1 M KOH Cool down to 30°C pH 8.5 with 0.1 M KOH, steril filtered
SSC (20×)	3 M NaCl 0.3 M Na-citrate pH 7 with NaOH
SSPE (20×)	3 M NaCl 0.2 M NaH_2PO_4 20 mM EDTA pH 7.4 with NaOH
STE (2×)	100 mM NaCl 20 mM Tris-HCl (pH 8) 10 mM EDTA 10 mM Tris-HCl (pH 8)
STET	10 mM Tris-HCl (pH 8) 100 mM NaCl 1 mM EDTA 5% (v/v) Triton X-100
Substrate solution (GUS-staining)	2 mM 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc, BIOSYNTH) in 50 mM-Na-Phosphate buffer (pH 7)
TE (pH 5.6)	10 mM Tris-HCl (pH 5.6) 1 mM EDTA (pH 8)
TE (pH 8)	10 mM Tris-HCl (pH 8) 1 mM EDTA (pH 8)

Name	Recipe
TTE (20×)	1.78 M Tris 0.57 M Taurin 2.7 mM EDTA
W5 (petunia assay)	154 mM NaCl 125 mM $CaCl_2$ 5 mM KCl Osmolarity: 0.6 osmol, steril filtered
Washing solutions (Southern)	SSPE (2×) + 0.5% (w/v) SDS SSPE (0.2×) + 0.1% (w/v) SDS

4.1.10 Media

Tab. 11. Media employed in this work

Name	Recipe
Gamborg's B5 medium (petunia cultivation)	0.31% (w/v) Gamborg's B5 Basal Salt Mixture (Sigma) 2% (w/v) Sucrose (Duchefa) pH 5.8 with KOH 0.8% (w/v) Purified Agar (Sigma)
LB medium (*E. coli* cultivation)	1% (w/v) bacto-tryptone 0.5% (w/v) bacto-yeast extract 172 mM NaCl pH 7 with NaOH
M9 medium (*E. coli* cultivation)	42.2 mM Na_2HPO_4 22 mM KH_2PO_4 8.5 mM NaCl 18.7 mM NH_4Cl pH to 7.4 with NaOH, autoclaving 1 M $MgSO_4$ (steril filtered) 20% (w/v) Glucose (steril filtered) 1 M $CaCl_2$ (steril filtered)
YT (2×) medium (*E. coli* cultivation)	1.6% (w/v) bacto-tryptone 1% (w/v) bacto-yeast extract 86 mM NaCl pH 7 with NaOH

4.1.11 Diverse materials

Tab. 12. Diverse materials employed in this work

Name	Company	Use
Agarose NuSieve GTG	Biozym	Electrophoresis of small DNA fragments
Agarose SeaKem LE	Biozym	DNA electrophoresis
Aquarium and swimmingpool synthetic filter wadding	Falke Agrar Zoo	Purification of DNA fragments from agarose gels
Cellulose Nitrate Membrane Filters (0.45 µm)	Whatman	GUS-staining assay
Diethylaminoethyl Cellulose (DE81)	Whatman	Gel retardation assay
Glass Fibre Prefilters	Sartorius AG	GUS-staining assay
Hybond^{TM}-N^{+} Nylon Transfer Membrane	Amersham-Pharmacia	Southern Blotting
Ion exchange chromatography paper	Whatman	Souther Blotting, Gel retardation assay
Magnetic Bio Beads Streptavidin	Merck	Elution of single stranded *Ds* DNA
Steril Filters (0.45 µm) Vacuflo PV 050/2	Schleicher & Schuell	Petunia transfection assay
Tissue Culture 12 ml steril Tubes	Greiner	Petunia transfection assay
Vitro Vent Steril Containers	Duchefa	Petunia seedlings culture

4.1.12 Diverse instruments

Tab. 13. Diverse instruments employed in this work

Instrument	Supplier Company
Centrifuge RC 5B *Plus*	Sorvall
Thermocycler	Gene E
RoboCycler Gradient96	Stratagene
Phosphorimager Storm 840	Molecular Dynamics
Pumpe Miniplus 2	Abimed Gilson
Spectrophotometer LambdaBio	Perkin Elmer
Scintillation Counter LS5000TD	Beckman
DNA sequencer	ABI Prism 377
Osmometer "Osmomat 030"	Gonotec
Ultracentrifuge L8-55M	Beckman
Varifuge 3.0 R	Heraeus Sepatech

4.2 METHODS

4.2.1 General molecular biology techniques

Unless stated otherwise, standard molecular biology methods like DNA gel electrophoresis, DNA restriction analyisis, DNA phenol/chloroform extraction, DNA ethanol/isopropanol precipitation, DNA phosphorylation and ligation, were performed according to Sambrook *et al.* (Sambrook *et al.*, 1989) or to the protocols provided by the enzyme suppliers.

For the purification of DNA fragments from agarose gel bands, aquarium synthetic filter wadding was used. By 10 min centrifugation at 13.000 rpm of the agarose gel band through the preautoclaved filter fiber in a 1.5 ml reaction tube at room temperature, the DNA containing liquid phase was down-centrifugated, whereas the solid remnants were retained on the wadding. The DNA was recovered by isopropanol precipitation. When cleaner DNA was needed, gel filtration through glass fiber matrix (GFX) micro spin columns was employed, as recommended by the manufacturer.

The concentration of double- and single-stranded DNA was photometrically measured with a Perkin Elmer LambdaBio-Spectrophotometer.
1 A_{260} (with 1 cm way) = 50 µg/ml dsDNA or 38-40 µg/µl of ssDNA.

4.2.2 Cultivation of various organisms

4.2.2.1 *E. coli*

The *E. coli* strains DH5α (used for plasmid cloning procedures and plasmid propagation) and JM110 (used for plasmid propagation) were grown in LB-medium according to Sambrook *et al.* (Sambrook *et al.*, 1989). Ampicillin was added when required at 100 mg/l. Bacteria were grown at 37°C. Details on the growing conditions of the *E. coli* strain NM552 are reported afterwards on session 4.2.6.2, Step 1.

4.2.2.2 *Petunia hybrida*

Petunia seeds were surface sterilized 2 min with 70% ethanol and 10 min with a SDS/Sodiumhypochlorite solution (0.2% (w/v) SDS, 10% (v/v) concentrated Sodiumhypochlorite). The seeds were then washed twice in steril water for 5 min and left to dry over night under the steril bench. Sterilized and dryed seeds were plated on Gamborg's B5 medium in Petri dishes and left to germinate under steril conditions at 25°C under a 16 hr light/8 hr dark cycle, in a climatic chamber (KBK/LS 4600, EHERET). 10-12 days old seedlings were transferred from Petri dishes to Vitro Vent Steril Containers and grown further for 3-4 weeks under the same medium, temperature and light conditions.

4.2.3 Transformation of various organisms

4.2.3.1 *E. coli*

The *E. coli* strains DH5α and NM552 were transformed with use of the $CaCl_2$/Heatshock-method (Cohen *et al.*, 1972). Competent cells preparation was based on the Rubidiumchloride-method according to Hanahan (Hanahan, 1983). The strain JM110 was transformed by electroporation as described by Sambrook *et al.* (Sambrook *et al.*, 1989). After transformation bacteria were plated on LB-medium supplemented with the appropriate antibiotic and incubated over night at 37°C.

4.2.3.2 *Petunia hybrida*

<u>Isolation of protoplasts from petunia mesophyll</u>

The entire procedure for protoplast isolation and transfection was accomplished under steril conditions. From totally 20-25 plants (3-4 weeks old) approximately 3 to 4 young leaves were harvested from each plant and transferred to a steril Petri dish. They were individually coarsely incised with a scalpell with 8-10 cuts perpendicular to the main vein. They were plasmolysed over night at room temperature in the dark in 30 ml steril filtered K3 medium containing 0.45 g Cellulase and 0.15 g Macerozyme. After digestion, protoplasts were swirled every 10 min for totally 30 min. By use of steril 10 ml wide tip plastic pipettes (such pipettes were used also in the next steps, every time protoplasts were

washed, resuspended, collected or transferred) protoplasts were collected and transferred to a sieve. By two consecutive filtrations through a 0.5 µm and a 0.25 µm stainless steel sieve, previously autoclaved, protoplasts were separated from undigested tissue. Protoplasts were then collected in aliquots of 10 ml suspension in totally 3×12 ml steril tissue culture tubes and centrifuged 10 min at 1000 rpm at room temperature with a Varifuge 3.0 R (Heraeus Sepatech) centrifuge. Sedimented cell remnants and the supernatant clarified K3 medium were sucked up with a glass capillary tube. Floating protoplasts collected in the bottom of the tube were washed with 16 ml W5-medium shared in 2×12 ml steril tissue culture tubes totally. After 10 min centrifugation at 600 rpm at room temperature, the sedimented protoplasts were resuspended in totally 10 ml W5 medium and stored in the dark for 90 min at 4°C in 1×12 ml steril tissue culture tube. Before storage step at 4°C, a 200 µl aliquot of the 10 ml protoplast suspension was added to 800 µl of W5-medium in 1.5 ml eppendorf tube and used to determine the number of total isolated protoplasts.

PEG-transfection of petunia protoplasts

After incubation at 4°C, protoplasts were collected by 10 min centrifugation at 600 rpm at room temperature, and resuspended in Ma-Mg transfection solution at a concentration of 1×10^6 protoplasts/300 µl solution. Aliquots of 300 µl of the protoplast suspension were transfected according to the $Ca(NO_3)_2$-PEG-method described by Negrutiu *et al.* (Negrutiu *et al.*, 1987).

Unless stated otherwise, when protoplasts were cotransfected with a *Ds* reporter plasmid and the TPase expression vector, 10 µg of each plasmid type were added to 40 µg of sonified CT-DNA (calf thymus DNA). Steril bidest water was added to a final volume of 60 µl. Plasmid DNA employed for protoplast transfection was propagated either in the *E. coli* strain DH5α (when *dam*-methylated DNA was needed) or in JM110 (when unmethylated DNA was needed). Plasmid DNA was isolated by use of the Plasmid Maxi-Kit of Qiagen and further purified by CsCl-gradient centrifugation and resuspended in 1× TE buffer (pH 5.6).

The DNA solution was pipetted to the 300 µl protoplast suspension already aliquoted in a 12 ml steril tissue culture tube. After the addition of 300 µl of 40% PEG_{6000}-solution and incubation at room temperature for 14 min, 5 ml W5 medium were dropped to each

transfection. Protoplasts were collected by 10 min centrifugation at 600 rpm at room temperature, carefully resuspended in 5 ml K3 medium and incubated at 26°C in the dark in Petri dishes.

Histochemical glucuronidase assay

The histochemical assay was performed essentially as described by Jefferson and coworkers (Jefferson *et al*., 1987) for tissue sections. The method allows the detection of the ß-glucuronidase expression in each transfected petunia cell. The hydrolase activity of the ß-glucuronidase can be recognized by incubation with the substrate X-Gluc: through oxidative dimerization of the reaction product (a indolyl derivative), an unsoluble, dark blue dye is generated that precipitates in correspondence of the enzymatic activity. Single ß-glucuronidase-expressing cells can be easily identified as blue spots through fixation on Nitrate Cellulose Membrane Filters (NC-filters) and incubation with the substrate X-Gluc.
Steps of the protocol: NC-Filters (0.45 µm, ∅=4 cm) were wetted in protoplast fixation solution and dried on Glass Fibre Prefilters. 200 µl of each 5 ml K3 medium/protoplast suspension were collected on a nitrocellulose filter and fixed for 30 min at room temperature. The protoplast-coated surface of each NC-filter was then incubated 30 min in fixation solution. The filters were washed twice for 20 min in 50 mM-Na-Phosphate buffer (pH 7.0); afterwards they were wetted with 1 ml of the same buffer containing the indigogenic substrate X-Gluc (2 mM) and incubated at 37°C in sealed Petri dishes. The stained protoplasts were routinely counted after overnight incubation, with help of a dissecting microscope.
Normally two to three 200 µl-aliquots of protoplast suspension per 5 ml transfection sample were fixed on NC-filter. The remaining protoplasts were collected and frozen on liquid nitrogen and stored at -80°C for further DNA analysis.

4.2.4 DNA isolation from various organisms

4.2.4.1 Isolation of plasmid DNA from *E. coli*

• Mini preparations of Plasmid DNA from *E. coli* were performed using the "boiling lysate" methode (Sambrook *et al*., 1989). Bacteria of 1.5 ml fresh over night culture were collected by centrifugation (8000 rpm, 2 min, RT) on a bench centrifuge (Eppendorf). The

bacteria pellet was resuspended in 400 µl STET buffer containing 1% (w/v) lysozyme and afterwards incubated 45 sec at 95°C. The lysed bacteria cells and the denatured genomic DNA were centrifuged (14000 rpm, 10 min, RT) and removed from the supernatant plasmid solution with a steril toothpick. The plasmid DNA was precipitated with 40 µl Na-acetate solution (pH 5.2) and 420 µl isopropanol for 5 min at room temperature. After centrifugation (14000 rpm, 5 min, RT) the DNA pellet was washed once with 70% ethanol, centrifuged again and left to dry. The pellet was then resuspended in 100 µl 1× TE (pH 8) buffer containing 20 µg/ml of RNase A and shaked 10 min at 45°C. The DNA solution was stored at -20°C.

• For the purification of large amounts of plasmid DNA the Plasmid Maxi- or Plasmid Midi-Kit from Qiagen were employed.
Plasmid DNA employed for petunia transient transfection assay was further purified by CsCl-gradient centrifugation. To each 4 ml DNA plasmid solution 4 g CsCl were added. The solution was transferred into a tube (13×15 mm, Beckman) using a pipette with a long and narrow tip. 150 µl of an ethidium bromide solution (1%) were then added to the DNA-CsCl solution and immediatly mixed till the dye was homogeneously dispersed. The tubes were then calibrated and sealed. Centrifugation of the density gradients was performed by ultracentrifugation in a vertical rotor (VTi80, Beckman) at 55000 rpm and 20°C over night. After the centrifugation a large band consisting of the closed circular plasmid DNA was visible. The band was collected by holing with a needle the top of the tube to allow air to enter and inserting a hypodermic needle into the tube just below the band and parallel to it. The band was carefully sucked up and collected into a 15 ml steril tube. To remove the ethidium bromide from the DNA, an equal volume of isopropanol saturated with a CsCl solution was added to the solution of DNA. After vortexing and centrifugation at 3500 rpm (Varifuge 3.0 R, Heraeus Sepatech) for 3 min at room temperature, the lower aqueous phase was transferred to a clean tube. The extraction was repeated four to six times till the pink color disappeared. Finally, to the cleaned DNA solution 4 volumes of steril bidest water and 4 volumes of isopropanol were added and the DNA precipitated 15 min at room temperature. By centrifugation at 14000 rpm for 30 min at RT (Centrifuge RC 5B *Plus*, Sorvall) the DNA was pelleted. After a washing step with 70% ethanol, the DNA was dried and resuspended in 1× TE buffer, pH 5.6.

4.2.4.2 Isolation of total DNA from petunia protoplasts

At the timepoint the protoplasts were harvested for GUS-staining, the remnant protoplast solution was collected on a 12 ml steril tissue culture tube and centrifuged (12000 rpm, 10 min, 4°C, Centrifuge RC 5B *Plus*, Sorvall). The sedimented protoplasts were frozen in liquid nitrogen. Total DNA was isolated by use of the DNeasy Plant Mini Kit. The DNA was eluted in 100 µl elution buffer and stored at -20°C.
To avoid contamination of the DNA preparations with extracellular plasmid DNA, protoplasts were treated with DNase I prior to cell collection by adjusting the protoplast culture medium to 20 mM $MgCl_2$ and adding 1 µg/ml DNase I. The mixture was incubated for 30 min at 28°C. The reaction was stopped by the addition of EDTA (pH 8) to a final concentration of 50 mM. The cells were immediately collected and frozen in liquid nitrogen. DNA extraction was performed as described above.

4.2.5 DNA detection

4.2.5.1 Southern Blot analysis

Total DNA isolated from transfected protoplasts and restricted with the appropriate enzyme or total PCR reactions performed on protoplast DNA were separated by gel electrophoresis. The DNA fragments were transferred to a Hybond-N+ Nylonmembrane through alkaline-capillary-transfer (Southern, 1975), according to Sambrook *et al.* (Sambrook *et al.*, 1989). To improve the transfer of large DNA-fragments, the agarose gel was incubated in 0.25 M HCl for 25 min at RT before denaturation. Denaturation was performed in 0.4 M NaOH solution for 25 min at RT. After the transfer, the filter was dried and the DNA was covalently bound to the membrane under UV-light exposure.
Hybridization of DNA filters with α^{32}P-dCTP labeled gene specific probes was performed in hybridization solution over night at 65°C. This was accomplished after the filters were preincubated for 1 to 2 hr, in the absence of the probe, in the same solution and at the same temperature. Washing of the filters was performed under stringent conditions: a first wash in 2× SSPE + 0.5% SDS (15 min) was followed by three to four further whashes in 0.2× SSPE + 0.1% SDS (15 min each). Afterwards the filters were analysed by autoradiography

on X-ray films or by use of a Phosphorimager Storm 840. Quantification of the band-intensities was performed with the Tina 2.09g software, Raytest (Straubenhardt).

Labeling of the probes

The labeling of the DNA probes was performed by random priming using the Ready To Go™ DNA Labeling Beads (-dCTP) from Amersham-Pharmacia, according to the indications of the manufacturer. At the end of the reaction, the labeled DNA was separated from the unincorporated nucleotides with use of the Sephacryl S-300 HR spin columns. The amount of ^{32}P-labeling was determined with a Scintillator Counter LS5000TD.

4.2.5.2 PCR analysis to study *Ds* excision frequencies

To amplify the empty *Ds* donor site (300 bp excision product), a polymerase chain reaction (PCR) was performed on 150 ng total DNA from transfected protoplasts using primers Pr.1 and Pr.2. With the same primer pair a 250 bp fragment was coamplified from the plasmid pNT150 which lacks the *Ds* insertion and that was added (1 pg) as a control to each reaction tube. The reaction mixture (50 µl) contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer and 2.5 units of *Taq* DNA polymerase.

After 5 min of initial denaturation at 95°C, 25 cycles of amplification were carried out (94°C, 30 sec; 60°C, 20 sec), followed by a final 5 min extension step at 72°C.

The PCR reaction was performed in the thermocycler "RoboCycler Gradient96" from Stratagene.

4.2.5.3 PCR analysis to study *Ds* excision footprints

Ds excision footprints were analysed by amplification of the empty *Ds* donor site (300 bp excision product) under the same PCR conditions described above (4.2.5.2.), except for the addition of the control plasmid pNT150 to the reaction mixture before amplification. PCR products were separated on 1% (w/v) gel electrophoresis and purified with use of aquarium synthetic filter wadding (see section 4.2.1.). Individual amplified molecules were cloned into pCR2.1-TOPO plasmid (TOPO TA Cloning® Kit, Invitrogen), according to the protocol provided by the manufacturer. The ligations were transformed into the *E. coli*

cells One Shot TOP 10F' (Invitrogen). Plasmid DNA was isolated from random bacteria colonies by use of the "boiling lysate"-methode. Through *Eco* RI digestion, positive clones containing the 300 bp empty *Ds* donor site were selected. Before sequencing, DNA mini preparations were extracted 2 to 3 times with phenol/chloroform and purified by ethanol precipitation. Sequencing of the footprints was carried out with the primer opUC>350 by use of a BigDye Terminator Cycle Sequencing Ready Reaction with AmpliTaq-Polymerase (ABI) on the DNA sequencer ABI Prism 377.
Total DNA from 4 independent petunia transfection assays was extracted and on each DNA preparation one to two polymerase chain reactions were done. Once each amplification product was ligated into pCR2.1-TOPO and the ligation product was transformed into *E. coli* cells, the plasmid inserts from 7 to 18 colonies were sequenced per transformation. Thus for each plasmid, 65 independent footprints were analysed in total.

4.2.6 Special methods for the preparation of holomethylated and hemimethylated DNA plasmid

4.2.6.1 Preparation of holomethylated *Ds* reporter plasmids

To generate holomethylated plasmids, pMiDsf1 and pMiDsf1-RI were treated with *Sss* I-Methylase (M-*Sss* I) for 16 hr at 37°. Each reaction mixture contained 2 μg of CsCl purified plasmid DNA, 50 mM NaCl, 10 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 1 mM DTT, 160 μM S-adenosylmethionine (SAM) and 2 units of M-*Sss* I. Every 4 hr the reaction was supplemented with fresh SAM. After phenol/chloroform extraction and ethanol precipitation, the DNA was resuspended in 1× TE (pH 5.6). The completeness of the reaction was confirmed by *Hpa* II (a CpG methylation sensitive restriction enzyme) restriction digestion. The mock-methylated plasmids were prepared under the same conditions except for the presence of M-*Sss* I in the reaction mixture.

4.2.6.2 Preparation of reporter plasmids with hemimethylated *Ds*

A shematic of the preparation of locally hemimethylated plasmid DNA is depicted in Fig. 20 in Results section 2.3.2.1. Details on the reaction conditions followed at each procedure step are indicated below.

Step 1. Isolation of phagemid single stranded DNA.

The *E. coli* host strain NM552 was grown and mantained onto M9 minimal medium containing 1 µg/ml thiamine to insure retention of F' episome which is required for infection by phage. NM552 cells transfected with the plasmids pMiDsf1, pMiDs<1f and pMiDsf1-RI were grown to A_{660} = 0.5-0.8 (1 A_{660} = 8×10^8 cells) in 2× YT medium supplemented with 0.001% thiamine and 150 µg/ml Ampicillin at 37°C. The density of bacteria liquid cultures was spectrophotometrically determined with a Perkin Elmer LambdaBio-Spectrophotometer.

2 ml of this culture were infected with the bacteriophage M13K07 at a multiplicity of infection (m.o.i.) of 10 and shaked, in a 50 ml tube, at 300 rpm for 1 hr at 37°C. 400 µl of the infected cells were mixed with 10 ml of 2× YT medium containing 70 µg/ml of kanamycin and grown, in a 50 ml Erlenmeyer flask, at 300 rpm for 14-18 hr at 37°C. The cells were then pelleted by centrifugation (12000 rpm, 15 min, room temperature, Centrifuge RC 5B *Plus*, Sorvall). To the retained supernatant, containing the single stranded DNA (ssDNA) "phage", 0.25 volume of a 3.5 M ammonium acetate/20% (w/v) PEG_{6000} solution were added. After chilling on ice for 30 min, the precipitated ssDNA was collected by centrifugation (13000 rpm, 30 min, 4°C, Centrifuge RC 5B *Plus*, Sorvall) and resuspended (when the supernatant was completely discarded) in 400 µl 1× TE buffer (pH 8). After three times phenol/chloroform extraction the ssDNA was ethanol precipitated and resuspended in 1× TE buffer (pH 8). Before employing through the next steps, the phagemid ssDNA was further purified by CsCl-gradient centrifugation following the same procedure used for the purification of double stranded plasmid DNA.

Step 2. PCR amplification of the *Ds* element

Amplification of the *Ds* element was carried out using the primers: bDs2 and mDs3, for the isolation of methylated *Ds* lower strand (pMiDsf1-*Hemi* and pMiDsf1-RI-*Hemi* constructs); Ds2.II and bDs3, for the isolation of methylated *Ds* upper strand (pMiDs<1f-*Hemi* construct); bDs2 and Ds3, for the isolation of unmethylated *Ds* lower strand (pMiDsf1-mock construct). Plasmid pMiDsf1 was used as template. The PCR cocktail (50µl) contained 20 mM Tris-HCl (pH 8.8), 7 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% (v/v) Triton X-100, 0.1 mg/ml nuclease-free BSA, 0.2 mM of each dNTP (dCTP was facultatively replaced by ^{5m}dCTP), 0.2 µM of each primer, 10 ng of plasmid

template and 1.25 units of *Pfu* DNA polymerase. After 5 min of initial denaturation at 95°C, 30 cycles of amplification were carried out (94°C, 30 sec; 55°C, 30 sec; 72°C, 1 min 55 sec), followed by a final 5 min extension step at 72°C. Amplification reaction were performed on a Thermocycler (Gene E). The PCR product was cleaned by gel filtration through a glass fiber matrix micro spin columns (GFX).

Step 3. Phosphorylation of *Ds* amplified product

After purification, aliquots of approximately 1.2 µg of *Ds* PCR product were phosphorylated in 50 µl reaction mixture containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA, 1 mM ATP and 20 units of T4 Polynucleotide Kinase for 1 hr at 37°C. To improve phosphorylation efficiency, prior to kinase addition the reaction mixture was heated for 5 min to 70°C and then chilled on ice.

Step 4. Elution of *Ds* single strand

The methylated or unmethylated single stranded *Ds* element was prepared by adsorption of the biotinylated and phosphorylated PCR product to streptavidin-conjugated magnetic beads. 70 µl of a magnetic streptavidine coated beads suspension (10 mg/ml) were transferred to a 1.5 ml tube. The beads were collected on one side of the tube with use of a magnetic rack (Merck). The storage buffer was discarded and the beads washed twice with 10× SSC buffer. After the second washing step, the washing buffer was discarded and the beads resuspended in an aliquot of PCR product containing 1.2 µg of DNA. An equal volume of 20 mM Tris-HCl (pH 7.5), 1 mM EDTA and 2 M NaCl buffer was added to the DNA/beads solution. The binding was performed for 30 min at room temperature with gentle rotation of the tube. Afterwards, the beads were collected and the clarified solution containing unbound DNA was discarded. After two washes in 10× SSC buffer, the beads were incubated in 50 µl 0.1 N NaOH for 8 min at room temperature. In this way the non-biotinylated DNA strand was eluted. The basic eluate was immediately neutralized by adding 0.5 volume of 0.2 M HCl and 77 mM Tris-HCl (pH 8).

Step 5. Hybridization

Methylated or unmethylated single stranded *Ds* element was desalted in Sephacryl micro spin columns (S-300 HR) after elution from the streptavidine beads and annealed in a

molar ratio 2:1 to the single stranded phagemid DNA in 50 mM NaCl, 10 mM Tris-HCl (pH 8) and 5 mM EDTA (2× STE buffer). The hybridization solution was heated for 3 min at 95°C and allowed to cool down to 25°C in 2 hr. The hybridization product was further purified by gel filtration through micro spin columns (S-400 HR).

Step 6. Filling-in reaction

Approximately 60 ng of hybridization product were filled-in in a 50 µl reaction mixture containing 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% (v/v) Triton-X-100, 0.1 mg/ml nuclease-free BSA, 0.05 mM of each dNTP and 1.25 units of *Pfu* DNA polymerase for 1 hr at 72°C on a Thermocycler (Gene E) with heated cover. For the preparation of totally 3 µg of plasmid DNA, the amount of construct employed in each protoplast assay, at least 160 unique filling-in reactions were necessary per plasmid. The entire volume of the 160 filling-in reactions was collected in a steril 50 ml tube and the DNA was extracted twice by phenol extraction and purified by ethanol precipitation. The DNA was resuspended in 0.5× TE buffer, pH 8.

Step 7. T4 DNA Ligase treatment

The nick remaining on each filled-in plasmid was closed by use of the T4 DNA ligase. Aliquots of approximately 0.3 µg of DNA were treated with 2 units of T4 DNA Ligase over night at 16°C, in a final volume of 50 µl. All the ligation reactions relative to one preparation of fresh 3 µg of "*Hemi-Ds*" construct (tested afterwards in protoplast transfection assay) were collected on a 1.5 ml tube. The DNA was extracted twice with phenol/chloroform and purified by ethanol precipitation. The DNA was finally resuspended in 1× TE buffer (pH 5.6) and stored at -20°C.

4.2.7 Preparation of differentially methylated *Ds* ends and 5' end segments for gel retardation assay

4.2.7.1 5' and 3' *Ds* ends

C-methylated and unmethylated 5′ and 3′ Ds ends

By PCR amplification of the *Ds* element in presence and absence of ^{5m}dCTP in the reaction mixture, respectively C-methylated and unmethylated *Ds* was prepared. Primers Ds2.II and mDs3 were employed for the amplification of the methylated element and primers Ds2.II and Ds3 for the unmethylated one. PCR conditions were the same applied for the amplification of the *Ds* element as described in section 4.2.6.2, step 2. After purification through a Sephacryl S-300 HR spin column, the PCR product (700 bp) was digested with the C-methylation resistant *Dra* I enzyme that cuts once within the *Ds* element. Two fragments 300 bp (*Ds* 5' end) and 400 pb (*Ds* 3' end) in length were generated that were separated by gel electrophoresis in 1.5% Agarose SeaKem and 1× TTE buffer. The gel bands were purified by gel filtration in glass fiber matrix (GFX) micro spin columns.

Hemimethylated 5′ and 3′ Ds ends

Hemimethylated *Ds* ends were constructed by the combinatorial hybridization of a methylated with an unmethylated *Ds* single strand and digestion of the hybridization product with *Dra* I. The two fragments of 300 bp and 400 bp corresponding to the *Ds* 5' end 3' end respectively were further purified by gel electrophoresis.
Methylated *Ds* strands were prepared by PCR amplification of the *Ds* element (section 4.2.6.2., Step 2) in presence of ^{5m}dCTP and primers bDs2 and mDs3 (for the isolation of methylated *Ds* lower strand) or Ds2.II and bDs3 (for the isolation of methylated *Ds* upper strand). The biotinylated PCR products were directly adsorbed to streptavidin-conjugated magnetic beads and the non-biotinylated methylated DNA strands were eluted with NaOH following the procedure already described in section 4.2.6.2., Step 4. Unmethylated *Ds* single strands were prepared in the same way like the methylated strands except for the presence of ^{5m}dCTP in the PCR reaction mixture; moreover, primers bDs2 and Ds3 (for the isolation of unmethylated *Ds* lower strand) and Ds2.II and bDs3 (for the isolation of unmethylated *Ds* upper strand) were in this case employed. The single stranded DNAs were hybridized in a molar ratio 1:1 in 2× STE buffer by heating the DNA solution for 3

min at 95°C and allowing to cool down to 25°C in 2 hr. Before digestion with *Dra* I, the hybridization products were purified using the Sephacryl S-300 HR spin columns.

4.2.7.2 *Ds* 5' end binding site blocks

Methylated, unmethylated and hemimethylated 5' binding site blocks ("I", "II", and "III" in Fig. 22 of Results section 2.3.2.3) were prepared by combinatorial hybridization of complementary methylated or unmethylated oligos corresponding to the *Ds* 5' end sequences: nucleotides 30-96 (OLIGO 1, OLIGO 1 MET, OLIGO 2, OLIGO 2 MET), 106-158 (OLIGO 3, OLIGO 3 MET, OLIGO4, OLIGO 4 MET) and 168-242 (OLIGO 5, OLIGO 5 MET, OLIGO 6, OLIGO 6 MET). Hybridization was performed as already described for the *Ds* ends (see above section 4.2.7.1, 'Hemimethylated 5' and 3' *Ds* ends'). The hybridization products were further separated from unhybridized single stranded DNA by gel electrophoresis in 1% (w/v) agarose SeaKem LE + 3% (w/v) agarose NuSieve GTG and 1× TTE buffer. The gel bands were purified by gel filtration in glass fiber matrix (GFX) micro spin columns.

4.2.7.3 Labeling of the probes

Approximately 100 ng of each DNA fragment were end-labeled in totally 30 µl reaction mixture containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA, 40 µCi γ^{32}P-ATP (6000 Ci/mmol) and 20 units T4 Polynucleotide Kinase. The reaction was incubated 45 min at 37°C and stopped by the addition of EDTA (pH 8) to a final concentration of 25 mM. The labeled DNA was separated from the free nucleotides by using the Sephacryl S-300 HR (5'/3' *Ds* ends) or G-25 (5' binding sites blocks) spin columns. The amount of ^{32}P-labeling was determined with a Scintillator Counter LS5000TD.

In the case of the 5' and 3' *Ds* ends, the DNA restriction fragments were dephosphorylated with use of Shrimp Alkaline Phosphatase (SAP) before labeling. The reaction mixture contained 400 ng DNA, 20 mM Tris-HCl (pH 8), 10 mM $MgCl_2$ and 0.3 units of enzyme in a final volume of 20 µl. The reaction was performed for 90 min at 37°C. Finally the samples were incubated 15 min at 65°C to denature the SAP.

4.2.8 Gel retardation assay (EMSA=electrophoretic mobility shift assay)

Gel retardation assays were performed using renatured $TPase_{103-465}$ (kindly provided by Ruth Adolphs) essentially as already described (Feldmar and Kunze, 1991; Becker and Kunze, 1997). Approximately 0.4 ng (15000-20000 cpm) of γ^{32}P-ATP labeled DNA fragments were incubated with renatured $TPase_{103-465}$ in 1× EMSA-binding buffer with 5 µg poly(dI-dC) in a total reaction volume of 30 µl for 20 min at 25°C. Subsequently the samples were subjected to gel electrophoresis in EMSA-electrophoresis buffer. A 0.8% and a 1.2% (w/v) gel (agarose SeaKem LE) were used for the gel retardation assay of the 5'/3' *Ds* ends and the 5' binding site blocks respectively. The electrophoretic migration was performed at 90 V for 3 hr and 1 hr for the 5'/3' *Ds* ends and the 5' binding site blocks respectively. The gels were then vacuum dried at 70°C onto cellulose paper and the radioactivity was detected by autoradiography or through the Phosphorimager.

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