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Ac-induced disruption of the double*Ds* structure in tomato

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Summary. The maize double*Ds* element is stably maintained in the tomato genome. Upon the subsequent introduction of *Ac* into a plant containing double*Ds*, disruption of the double*Ds* structure and DNA rearrangements at the site of the double*Ds* element were observed. No indications were obtained for excision of the complete double*Ds* structure. The consequences of transactivation of double*Ds* in these experiments are different from those described for transactivation of single *Ds* elements in tomato. The mechanisms by which such rearrangements could have occurred in tomato are discussed in relation to complex insertions containing double*Ds* in maize.

Key words: Transposable elements – Double*Ds* – *Ac/Ds* – Transgenic tomato – DNA rearrangements

Introduction

The maize double*Ds* element is, both in structure and in function, one of the most complex *Ds* elements characterized until now. Double*Ds* consists of two identical *Ds* elements with a size of 2040 bp (Döring et al. 1984). One of the elements is inserted into the approximate centre of the other in inverse orientation. Therefore, the double*Ds* structure consists of sequences which are present as inverted repeats.

The behaviour of double*Ds* has been examined with genetic and cytogenetic techniques in maize. These studies showed that the double*Ds* element can transpose, i.e. excise from its original position and reintegrate elsewhere in the genome (McClintock 1948). This property, shared by all *Ds* elements, is under the control of the maize *Ac* element. Unlike other *Ds* elements, transposition of double*Ds* can be accompanied by duplication of DNA segments or can otherwise result in chromosomal rearrangements (McClintock 1950). Furthermore, double*Ds* can be a site for chromosome breakage (McClintock 1945). Although the occurrence of this

event has been well documented, induced breakage at double*Ds* has not yet been molecularly analyzed. Such an analysis is complicated by the abundance of maize DNA sequences resembling double*Ds* (Fedoroff et al. 1983).

Some insight into the possible mechanisms which lead to rearrangement or breakage has been obtained from the molecular analysis of two unstable maize alleles which contain double*Ds* structures. These alleles carry insertions with a size of 30 kb (*sh-m5933*) and 45 kb (*sh-m6258*), respectively. The insertions are partly inverted duplications with double*Ds* structures at the ends of the duplicated segment. The origin of both insertions can be explained by postulating that cleavage occurred correctly at only one terminus of the double*Ds* element while the second cleavage occurred at a distance of several kilobases within the flanking DNA (Fedoroff 1989; Döring et al. 1990).

To facilitate the analysis of the behaviour of double*Ds*, this element was introduced into tomato. It is shown that double*Ds* is stably maintained in the tomato genome. Furthermore, evidence is provided that the subsequent introduction of *Ac* into a double*Ds*-containing plant leads to alterations at the double*Ds* structure. These rearrangements will be discussed in relation to the structures of the maize *sh-m5933* and *sh-m6258* alleles.

Materials and methods

Construction of plant vectors. The binary plasmid vector pTT250, which was used to introduce double*Ds* into tomato, contains, apart from double*Ds*, the selectable marker genes encoding hygromycin phosphotransferase (HPT II) and neomycin phosphotransferase (NPT II). The vector was constructed in two steps. First, a 1 kb *Bam*HI fragment containing HPT II was inserted into the *Bam*HI site of the expression cassette pRok1 (Baulcombe et al. 1986). After filling-in the *Bam*HI site between HPT II and the polyadenylation signal, the resulting plasmid was named pTT212. Then, plasmid
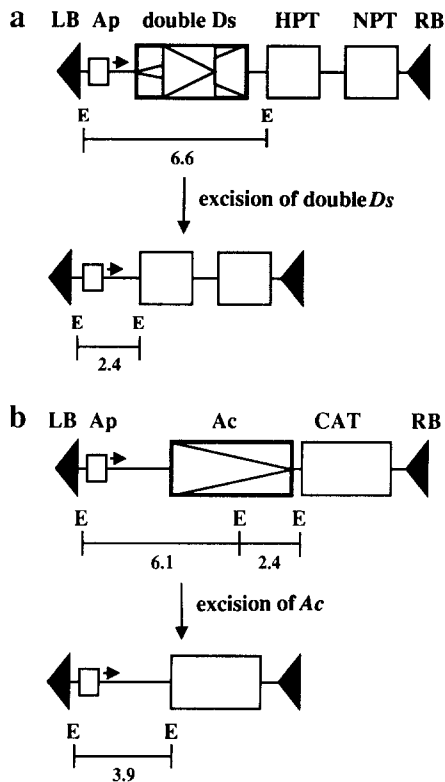
Fig. 1 a and b. Structure of the T-DNA region of pTT250 (a) and pTT252 (b) and the predicted structure of these regions upon excision of double*Ds* and *Ac*, respectively. The *arrow* represents the origin for replication of pBR322. LB, left border; RB, right border; E, *Eco*RI; Ap, ampicillin resistance gene; HPT, hygromycin phosphotransferase gene; NPT, neomycin phosphotransferase gene; CAT, chloramphenicol acetyltransferase gene

pBRK218, containing double*Ds* isolated from the maize allele *sh-m5933* (Döring et al. 1984) was inserted into the *Eco*RI site near the left border of pTT212. The T-DNA region of this plasmid is presented in Fig. 1a. In this diagram, the expected empty donor site fragment for double*Ds* is indicated as well.

The binary vector which was used to introduce *Ac*, designated pTT252, was derived from a vector containing NPT II and a functional chloramphenicol acetyltransferase (CAT) gene between the borders of the T-DNA (Haring and De Block 1990). The NPT II gene was disrupted by deletion of a 1 kb *Pst*I fragment. Subsequently, the *Ac*-containing plasmid pJAC (Behrens et al. 1984) was inserted into the *Eco*RI site. Both the T-DNA region of pTT252 and the expected empty donor site fragment for *Ac* are presented in Fig. 1b.

Transformation of transgenic plants. The constructed binary vectors were introduced into *Agrobacterium tumefaciens* LBA4404 (Hoekema et al. 1983) by triparental mating (Fraley et al. 1985). The tomato genotype Msk9 (Koornneef et al. 1986) was transformed as described previously (Rommens et al. 1991).

Southern blot analysis. Genomic DNA was isolated from greenhouse-grown plants (Haring et al. 1989). Ten micrograms of DNA was digested with restriction enzymes according to the manufacturer's recommendations

(Bethesda Research Laboratories), separated by 0.8% agarose gel electrophoresis and transferred to Hybond N membranes (Amersham) by vacuum blotting with 10 × SSC (LKB). Hybridization with randomly primed labeled probes was carried out in 10% dextran sulphate (Pharmacia), 1 M NaCl, 1% SDS and 200 µg/ml denatured salmon sperm DNA at 60° C. After washing down to 0.1 × SSC at 60° C, blots were autoradiographed using Kodak X-omat AR films. Before reprobing, filters were stripped with 0.4 N NaOH and neutralized with 1 × SSC, 0.1% SDS and 0.2 M TRIS-HCl at a pH of 7.5.

Three fragments of *Ac* were used as probes to allow the identification of, and discrimination between, genomic DNA fragments containing *Ac* or double*Ds*. These fragments were isolated from pJAC (Behrens et al. 1984) as a 4.3 kb *Bam*HI-ClaI fragment containing almost the entire *Ac* sequence (designated Ac), a 2.2 kb *Acc*I-*Hinc*II fragment containing an internal *Ac* sequence which is not homologous to double*Ds* (intAc) and a 1.5 kb *Bam*HI-*Hind*III fragment from the 5' side of *Ac* (frAc). Apart from the *Ac* fragments, the ampicillin resistance gene (Ap, ·a 1.1 kb *Bgl*I fragment from pUC19) and the HPT II gene (Gritz and Davies 1983) were used as probes.

Plasmid rescue. Part of our study on the behaviour of double*Ds* and *Ac* in transgenic tomato plants was carried out by restriction mapping of rescued circularized genomic DNA fragments. To prepare DNA for this purpose, 20 µg of an *Eco*RI digest was circularized in 1 ml of ligase buffer (BRL) in the presence of 10 units T4 DNA ligase (Pharmacia). To prepare electrocompetent cells, 200 ml of an *Escherichia coli* NM554 culture (Raleigh et al. 1988) was grown to early log phase ($OD_{600} = 0.5$) in LB broth and then pelleted by centrifugation (4000 rpm for 10 min at 4° C). Subsequently, cells were washed twice in distilled water at 4° C (in 1 volume and 1/5th volume, respectively) and then washed in 1/20th volume of 10% glycerol. After the final centrifugation, approximately 3×10^{10} cells were resuspended in 400 µl of 10% glycerol and 10% polyethylene glycol (molecular weight = 3350) and stored in 40 µl aliquots at −70° C.

To determine the transformation efficiency, 40 µl of electrocompetent cells were mixed with 1 pg of pUC19 and subsequently electroporated with a Biorad Gene Pulser (12.5 kV/cm for 5 ms). Next, cells were grown (by incubating cells with 1 ml of LB broth at 37° C for 30 min) and plated on agar plates containing 100 mg/l ampicillin for selection of transformants. If the transformation frequency was between 5×10^8 and 2×10^9 transformants/µg of plasmid DNA, electrocompetent cells were used for the recovery of genomic DNA fragments. Under these conditions, up to 50 circularized DNA fragments containing Ap and the origin for replication of pBR322 could be recovered per 20 µg of genomic plant DNA.

Sequence analysis. DNA sequences were determined from double-stranded plasmid DNA with Sequenase Version 2.0 (United States Biochemicals) according to the manufacturer's protocol.

Results

Stability of doubleDs in tomato

Tomato plants were transformed with an *Agrobacterium* strain harbouring the binary plasmid vector pTT250 (Fig. 1a). This vector contains bacterial plasmid sequences next to the double*Ds* element within its T-region. The presence of the bacterial origin sequences enables the structure of the T-DNA insertion to be analyzed by plasmid rescue. Also, the NPT II and HPT II genes are present between the borders of the T-region. The NPT II gene allows selection for transformants; the HPT II gene was not used in the present experiments.

The maize double*Ds* element contains two *Ds* elements which are in inverted repeat configuration. Therefore, this structure might give rise to events like recombination in tomato. To ascertain whether double*Ds* is stably maintained in tomato, four independent transformants were analyzed. A Southern blot, prepared from *Eco*RI-digested DNA was probed with Ac, stripped and subsequently reprobed with Ap. The size of the single fragment which was visualized on this blot is 6.6 kb and is identical to the size of an internal T-DNA fragment which contains Ap and double*Ds* (Fig. 2). This result was confirmed using *Bam*HI digests (data not shown). No additional signals were observed on the Southern blots even after prolonged exposure times, indicating that no major rearrangements occurred at the site of double*Ds* at this stage.

Characterization of a transgenic tomato plant containing a single doubleDs element and a mobile Ac element

A transgenic tomato plant, designated AAT1915, contains one T-DNA insertion of pTT250. This was deduced from Southern blots prepared from *Hin*dIII-digested plant DNA. One band showing homology with Ap, double*Ds* and HPT II represents the left border fragment. This band has a size of 18 kb (Fig. 3a, lanes 1). Another band, with a size of 20 kb, contains the NPT II gene and represents the right border fragment (data not shown). Reasoning that the presence of one double*Ds*-containing T-DNA insertion will facilitate the analysis of the behaviour of double*Ds* in the presence of *Ac*, plant AAT1915 was selected for the subsequent introduction of *Ac*. For this purpose, an *Agrobacterium* strain containing pTT252 (Fig. 1b) was used. Like pTT250, this vector contains sequences between the borders of the T-region which can be used for plasmid rescue experiments. The presence of the CAT gene in this T-region allowed double transformants to be selected for on chloramphenicol.

The behaviour of double*Ds* was studied by comparing the hybridization patterns of DNA isolated from AAT1915 and from a double transformant containing a mobile *Ac* element, designated AAT1915-24. In Fig. 3a, a Southern blot prepared from *Hin*dIII digests and probed with several constituents of the T-DNA region is presented. From the Southern blot, it can be deduced that *Ac* is predominantly present at its original
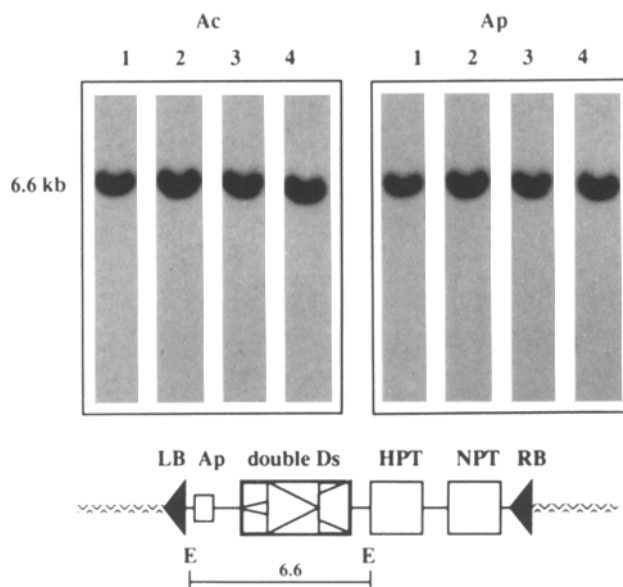

Fig. 2. Stability of double*Ds* in transgenic tomato plants. A Southern blot containing *Eco*RI-digested DNA isolated from four independent transformants (lanes 1–4) was probed with Ac, stripped and reprobed with Ap. The size of the observed fragment is identical to the size of an internal fragment of the T-DNA region of pTT250 containing Ap and *Ac*. E, *Eco*RI

position between the borders of the T-DNA. This is evident from the presence of a 1.3 kb band and a 16 kb band on probing with Ac (lane 2). The 16 kb band hybridizes also to Ap and weakly hybridizes to the intAc probe (due to the small overlapping sequence (100 bp) between fragment and probe; lanes 2). An additional 1.6 kb band is homologous to Ac and represents an internal *Ac* fragment.

The presence of an empty donor site fragment for Ac in the genome of AAT1915-24 indicates that *Ac* is active in this plant. The empty donor site fragment, with an expected size of 14.3 kb (16 kb minus a 1.7 kb *Hin*dIII fragment containing *Ac*), is difficult to detect on this blot but can more easily be visualized as a 3.9 kb band on Southern blots prepared from *Eco*RI digests (data presented in the next section).

Disruption of the doubleDs structure in the presence of Ac

Compared to the relative stability of *Ac*, double*Ds* is very unstable. This is evident from our inability to detect the full donor site fragment for double*Ds* in DNA of AAT1915. As mentioned previously, this fragment is present in DNA of AAT1915 as an 18 kb band. It contains, apart from double*Ds*, sequences flanking double*Ds* at the left side (Ap) and sequences flanking double*Ds* at the right side (HPT II). Instead of the original 18 kb fragment, two new fragments containing double*Ds* sequences (i.e. bands hybridizing to Ac but not to intAc) can be detected on the blot prepared from DNA of AAT1915-24 (Fig. 3a, lanes 2). These fragments have a size of 13 kb and 5.5 kb, respectively. The 13 kb fragment is also homologous to Ap but does not contain
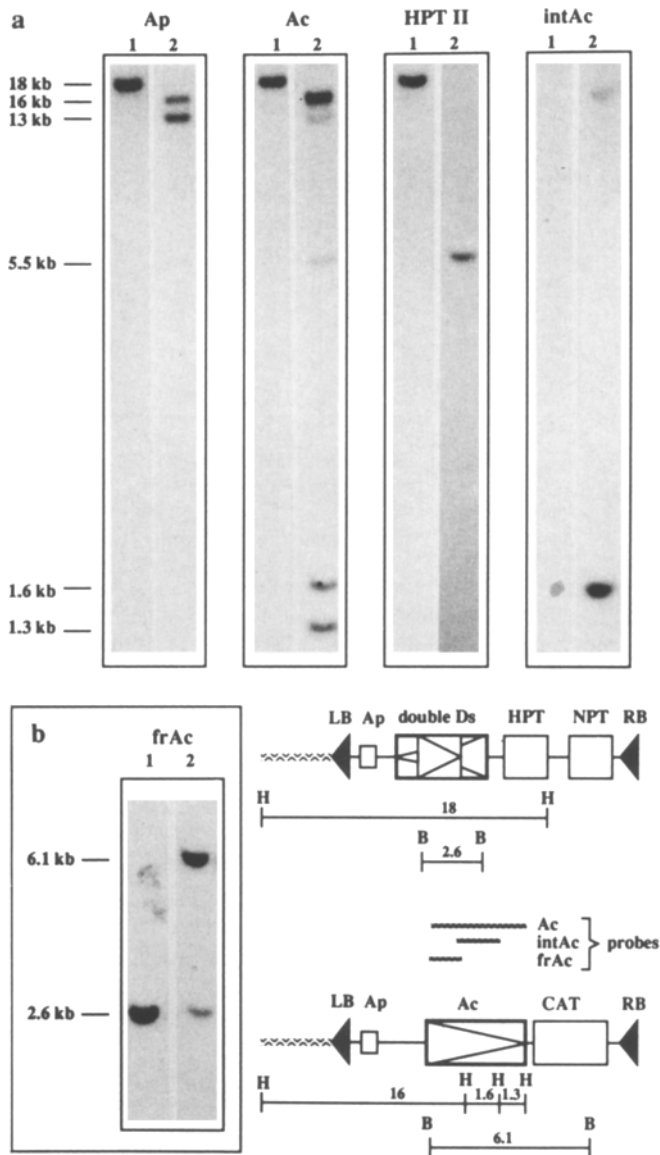
Fig. 3a and b. Behaviour of doubleDs in AAT1915-24. Southern blots were prepared from equal amounts (10 µg) of HindIII- (a) or BamHI- (b) digested DNA isolated from AAT1915 (lanes 1) or AAT1915-24 (lanes 2). The Ac fragments which were used as probes are represented with a dashed line. Also, the HPT II gene was used as a probe. a The original 18 kb HindIII fragment hybridizing to Ap, Ac and HPT II cannot be detected in DNA of AAT1915-24. The disappearance of this band correlates with the appearance of two new predominant bands with sizes of 13 kb and 5.5 kb. The 13 kb band hybridizes to Ap and Ac while the 5.5 kb band hybridizes to Ac and HPT II. Since the separated sequences, which originally flanked doubleDs at the left and right side still hybridize to doubleDs, the DNA rearrangement probably occurred at this element itself. Several fragments which can be visualized in the DNA of AAT1915-24 represent the presence of Ac at its original position in the T-region of pTT252. These fragments have a size of 16 kb, 1.6 kb and 1.3 kb. As expected, the 16 kb fragment only weakly hybridizes to intAc. b The intensity of a 2.6 kb internal doubleDs fragment decreases after introduction of Ac, indicating that the presence of Ac results in disruption of the doubleDs structure. Probably, "transactivation" of doubleDs leads to cleavage at the internal terminus located between the two BamHI sites present in doubleDs. A 6.1 kb fragment in lane 2 represents the full donor site fragment for Ac in plant AAT1915-24. Fragment sizes are indicated in kb and were determined from λ DNA size markers

HPT II sequences. Conversely, the 5.5 kb fragment hybridizes to HPT II but does not contain Ap sequences. The origin of both fragments can be explained by postulating that Ac-induced cleavage occurred at the site of doubleDs, thus leading to disruption of this element. Additional support for this hypothesis was obtained by hybridizing a Southern blot containing BamHI-digested DNA with the frAc probe (Fig. 3b). This blot shows that the introduction of Ac leads to a strong decrease in intensity of an internal 2.6 kb doubleDs band, indicating that cleavage events occurred somewhere within this region.

Since AAT1915-24 is a primary (double) transformant, the occurrence of several independent events at doubleDs can be expected. However, apart from the clearly detectable disruption of doubleDs, the Southern blots presented provide no indications of other events at doubleDs. For instance, an empty donor site fragment for doubleDs cannot be visualized. Such a (HindIII) fragment should have a size of 13.8 kb (18 kb minus the size of doubleDs). Possibly, events other than the described disruption at doubleDs are not frequent enough to allow detection on Southern blots. Therefore, the technique of plasmid rescue was used to investigate the possible occurence of these rare events.

Plasmid rescue to analyse "transactivation" of doubleDs

Surprisingly, the full donor site fragment for doubleDs was recovered not only from EcoRI digests of AAT1915 (with high frequency) but also from digests of AAT1915-24 (though with lowered frequency; Fig. 4). This fragment has a size of 6.6 kb and can be visualized on a Southern blot prepared from DNA of AAT1915 (Fig. 2) but not on a similar blot prepared from DNA of AAT1915-24 (Fig. 4). This result indicates that rescue experiments are, indeed, a more sensitive tool than Southern blot analysis for the examination of chimaeric plants.

As expected, both the 6.1 kb full and the 3.9 kb empty donor site fragment for Ac were rescued from the genome of AAT1915-24 as well. These fragments can also be visualized on the corresponding Southern blot (Fig. 4).

Based on Southern blot experiments, the recovery of a 7.8 kb EcoRI fragment was also expected. This fragment is homologous to Ap (Fig. 4) and doubleDs (i.e. that the fragment hybridizes to Ac but not intAc; data not shown). However, the 7.8 kb fragment could not be rescued from the genome of AAT1915-24. Also, attempts to rescue the corresponding 13 kb Hind-III fragment were unsuccessful (data not shown). These experiments indicate that the region occupied by doubleDs and pBR322 sequences might not contain the complete functional Ap gene (see Discussion).

Apart from the previously described fragments, a 2.9 kb fragment was rescued from the genome of AAT1915-24. This fragment, which could not be detected on Southern blots, contains the sequence flanking doubleDs at the left side. However, on the right side, it does not contain any other constituents of the T-DNA
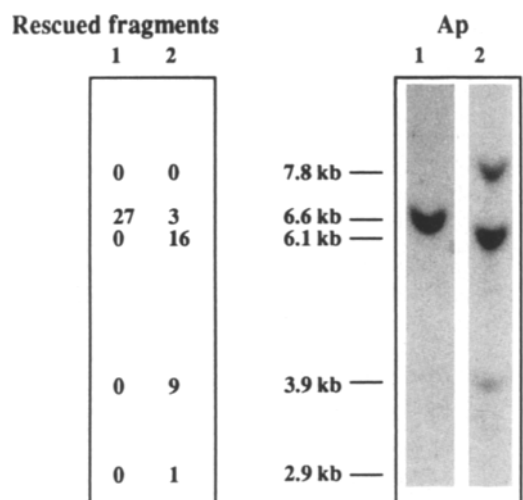
Fig. 4. Plasmid rescue from transformants AAT1915 and AAT1915-24. The table on the left shows the total number of independent *Eco*RI fragments which were recovered from the genomes of AAT1915 (lane 1) and AAT1915-24 (lane 2). A Southern blot prepared from similar DNA digests and probed with Ap is shown at the right. A comparison between the table and the autoradiogram of the blot demonstrates that fragments which were rescued with a low frequency from the genome of AAT1915-24 cannot per se be visualized on the blot. The fragments which were not visible on the autoradiogram (lane 2) have sizes of 6.6 kb and 2.9 kb

Fig. 5a and b. Analysis of a 2.9 kb *Eco*RI fragment rescued from the genome of AAT1915-24. **a** Comparison of sequences at the point of divergence between a 6.6 kb fragment rescued from AAT1915 and the 2.9 kb fragment recovered from AAT1915-24. The point of divergence, indicated with a *zig-zag line*, is present at 9 bp to the left of the original site of double*Ds*. Plant DNA sequences are indicated with a *thick dashed line*. **b** A Southern blot was prepared from *Hind*III-digested DNA of a non-transformed tomato plant. The blot was hybridized with the plant DNA fragment flanking the left border of the T-DNA (lane 1), stripped and reprobed with the plant DNA sequence present in the 2.9 kb plasmid (lane 2). Hybridization patterns show that the fragments are homologous to repetitive and single copy plant DNA sequences, respectively. E, *Eco*RI

(Fig. 5). Instead, it contains a single-copy plant DNA sequence. This was demonstrated by hybridizing this sequence with genomic DNA of non-transformed tomato plants (Fig. 5b, lane 2). The plant DNA sequence starts at 9 bp from the original site of double*Ds* (Fig. 5). This implies that the 2.9 kb rescued fragment derives from a DNA rearrangement which occurred at double*Ds*. In contrast to the previously described disruption of double*Ds*, the rearrangement probably originated from a cleavage event at, or near, the end of double*Ds*.

The plant DNA fragment fused to the sequence flanking double*Ds* at the left side is not related to the plant DNA fragment which flanks the right border of the T-DNA insertion. The latter fragment, rescued from DNA of AAT1915 digested with *Bam*HI and *Bgl*II, hybridizes to repetitive plant DNA sequences (Fig. 5, lane 1).

Discussion

In this report we demonstrate that the introduction of *Ac* into a tomato plant containing double*Ds* leads to DNA rearrangements at the double*Ds* structure. The fact that stability of double*Ds* in tomato plants which do not contain *Ac* could be demonstrated as well, implies that the observed rearrangements in AAT1915-24 are a consequence of transactivation of double*Ds* by *Ac*.

Previous reports have shown that single *Ds* elements can also be transactivated by *Ac* in tomato (Lassner et al. 1989). Transactivation of these *Ds* elements leads to excision of the element from its original position, an event which is commonly followed by reintegration of the *Ds* element elsewhere in the genome, as has now
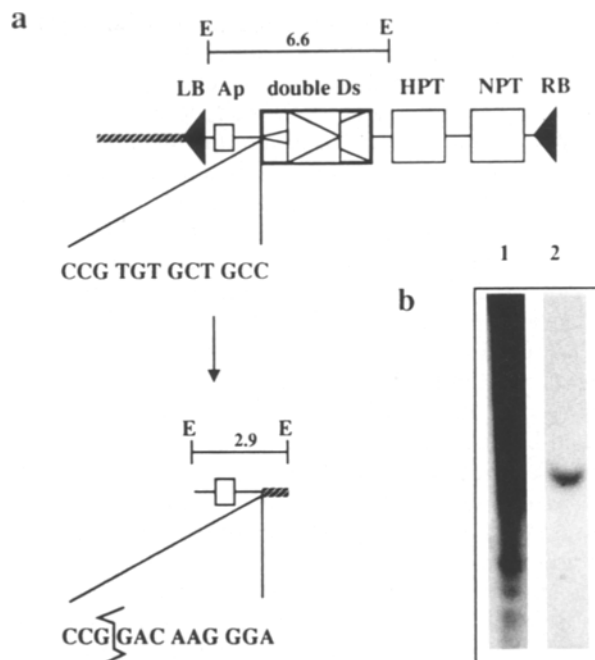
been documented in many plant species (for a review, see Haring et al. 1991). Excision of double*Ds*, however, could not be demonstrated in the genome of AAT1915-24. Therefore, although the ability of double*Ds* to excise in other tomato plants cannot yet be excluded, our studies show that the behaviour of double*Ds* in tomato is different from that of single *Ds* elements.

In most of the transgenic tomato plants containing *Ac* and *Ds* that have been analyzed, until now the full donor site of *Ds* is comparable in hybridization intensity to the empty donor site (Lassner et al. 1989; C.M.T. Rommens, unpublished results). In plant AAT1915-24, the full donor site of double*Ds* is not detectable on Southern blots. This suggests that either the timing of transactivation of double*Ds* is earlier during plant development or that the frequency of late transactivation events is higher than that common for single *Ds* elements. Because only one plant was examined in detail, it is not clear whether this phenomenon is a general feature.

Instead of excision of double*Ds* from its original location, different rearrangements were observed in the genome of AAT1915-24. The predominant rearrangement implies disruption of the double*Ds* structure: two new

fragments, still containing one side of doubleDs, each hybridize only with one of the doubleDs flanking sequences. Disruption of doubleDs is possibly due to the configuration of this element, which potentially leads to complex secondary structures upon transactivation.

Excision of single Ds elements is a consequence of cleavage events at both ends of the element. Since doubleDs consists of two Ds elements, each of which contains two terminal sequences, the cleavage events at doubleDs can occur at any of these four terminal repeats. The observation that disruption of doubleDs correlates with a strong decrease in intensity of an internal BamHI fragment of doubleDs suggests that one cleavage occurred at a terminus of the inner Ds element, which is located between the two BamHI sites of doubleDs.

A transactivation event which is restricted to cleavage events within doubleDs, followed by common repair processes, cannot account for the observed rearrangements present in plant AAT1915-24. The observed DNA rearrangements in this plant suggest that cleavage events at doubleDs coincide with additional cleavage events in flanking DNA. A similar hypothesis has been proposed to explain the origin of the maize sh-m5933 and sh-m6258 alleles (Döring et al. 1989; 1990; Fedoroff 1989). There are no clear indications for the positions of the cleavage events outside doubleDs in the tomato genome. However, the inability to rescue DNA fragments containing a disrupted doubleDs structure and Ap (a 7.8 kb EcoRI fragment or a 13 kb HindIII fragment which can be visualized on Southern blots) suggests that, with respect to this event, an additional cleavage occurred within the Ap gene.

Another rearrangement, examined by analyzing a rescued 2.9 kb EcoRI fragment, contains a sequence originally flanking doubleDs at the left side, fused to a plant DNA sequence. The origin of this rearrangement can be explained, as in the previous case, by postulating that cleavage at the left terminus of doubleDs coincides with cleavage to the right of the T-DNA insertion, within plant DNA. This would lead to loss of the intervening sequence containing the right part of the T-region. Alternatively, this rearrangement could have occurred via a mechanism which is different from that associated with the two major events causing disruption of doubleDs. It can be envisaged that, in this case, a primary cleavage event occurred on the left terminal repeat of doubleDs, but that transposition of doubleDs was aberrant, leading to a deletion at the site of excision. The occurrence of a deletion coinciding with an aberrant transposition event has been described previously in maize for the Ds1 element (Peacock et al. 1984).

The finding that doubleDs can be transactivated in tomato and that transactivation leads to events which are compatible with those observed in maize, justify a further examination of the behaviour of doubleDs in tomato. Further experiments may contribute to an understanding of the mechanism of transactivation of doubleDs and Ds.

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