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Size does matter: Cre-mediated somatic deletion efficiency depends on the distance between the target *lox*-sites

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Abstract

Cre/*lox* recombination *in vivo* has become an important tool to induce chromosomal rearrangements like deletions. Using a combination of *Ds* transposition and Cre/*lox* recombination in two independent experiments on chromosomes 6 and 7 of tomato, two sets of somatic deletions up to a size of 200 kb were obtained. The efficiency of somatic deletion decreased with increasing deletion size. The largest germinally transmitted deletion had a size of only 55 kb. The results show that Cre-mediated deletion in somatic cells is less efficient when the *lox* sites are separated over larger distances. A further drop of the deletion efficiency after germinal transmission of the larger deletions can be explained by the probable loss of genes that are of vital importance to gametophyte function. Plasmid rescue of an 8.4 kb circularised deleted DNA showed that the Cre-mediated deletion takes place in tomato as expected. Since the circular Cre-deleted DNA could only be PCR amplified in plant cells where the deletion was not complete, the double-stranded DNA circle is assumed to be instable.

Introduction

Since the introduction of the Cre/*lox* system from bacteriophage P1 into plant cells by Dale and Ow (1990), it has become the best characterised and most widely used recombination system in both commercial plant biotechnology and basic plant research. The Cre/*lox* system is used to induce chromosomal inversions, translocations and deletions *in planta* (reviewed by Ow and Medberry (1995) and Gilbertson (2003)).

To study the efficiency of Cre-mediated deletion in tomato chromosomes in relation to the size of the deletions a tactic was used as proposed by Van Haaren and Ow (1993) that unites map based cloning and insertion mutagenesis strategies. The key to our approach is the T-DNA shown in Figure 1A, the D3 construct, which contains two *lox* sites, one of which in a transposable *Ds* element from maize. After introduction into the plant genome, this construct offers three opportunities for genetic studies. Firstly, it can be used for

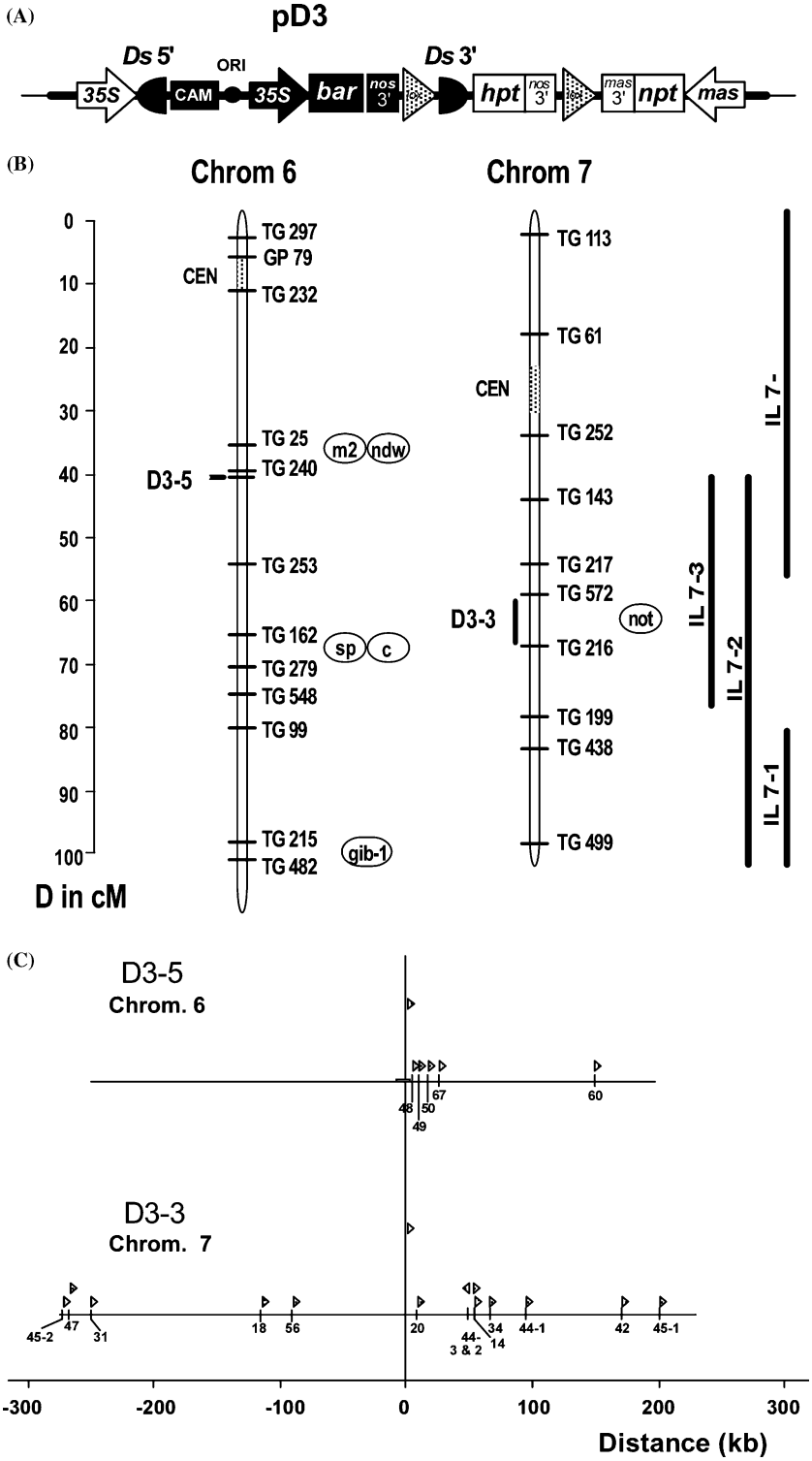

(A)
pD3
Ds 5'
ORI
Ds 3'
35S
CAM
35S
bar
nos 3'
hpt
nos 3'
mas 3'
npt
mas
(B)
Chrom 6
Chrom 7
0
10
20
30
40
50
60
70
80
90
100
D in cM
TG 297
GP 79
CEN
TG 232
TG 25
m2
ndw
D3-5
TG 240
TG 253
TG 162
sp
c
TG 279
TG 548
TG 99
TG 215
gib-1
TG 482
TG 113
TG 61
CEN
TG 252
TG 143
TG 217
TG 572
D3-3
not
TG 216
TG 199
TG 438
TG 499
IL 7-3
IL 7-2
IL 7-1
IL 7-
(C)
D3-5
Chrom. 6
48
49
50
67
60
D3-3
Chrom. 7
45-2
47
31
18
56
20
44-3 & 2
34
14
44-1
42
45-1
-300
-200
-100
0
100
200
300
Distance (kb)

Figure 1. (A) Schematic representation (not to scale) of the essential elements in the pD3 construct. The black coloured element, demarcated by the *Ds 5′* and *Ds 3′* borders of the *Ds* transposable element from maize, forms a functional transposable element that embodies the interjacent DNA. The rest of the T-DNA is depicted white, except for the grey triangles, which represent the *lox* sites. Furthermore, the constructs contain several *in planta* selectable markers: *npt*: the neomycin phosphotransferase II gene, *hpt*: the hygromycin phosphotransferase gene and *bar*: the Basta (Phosphinotricin) resistance gene. Promoter sequences are depicted as arrows; terminator sequences have a *3′* annotation. ORI represents a *Col E* bacterial origin of replication, CAM represents the chloramphenicol resistance gene for selection in *Escherichia coli* (enabling plasmid rescue), *35S* represents the Cauliflower Mosaic Virus *35S* promoter and *mas* elements of the Manopine Synthase gene, *nos* represents parts of the Nopalin Synthase gene, as indicated. The construction of pD3 is described in detail by Coppoolse *et al*. (2003). (B) Map positions of the D3-3 and D3-5 T-DNAs in the tomato genome. D3-5 is mapped on chromosome 6 in between markers TG 25 (D = 4.0 cM) and TG 253 (D = 12.0 cM). Crosses to lines carrying the *potato leaf* (*c*), the *mottled*-2 (m2), and *self pruning* (sp, present in line UC82B) genes confirmed the position on chromosome 6. The approximate map position of the *necrotic dwarf*, and *gibberellic acid deficiency*−1 loci are derived from Van Wordragen, *et al*. (1996). D3-3 is mapped on chromosome 7 in between RFLP markers TG 216 and TG 572, closely linked to the *Notabilis* gene (Burbidge, *et al*., 1999). The position of the centromer is derived from Van der Hoeven, *et al*. (2002). To confirm the mapping position of D3-3 on chromosome 7, the *Lycopersicon pennellii* introgression lines IL 7-1 to −4 (Eshed and Zamir, 1995) were used. The introgression segments are shown schematically at the right hand side of the panel. D3-3 flanking sequences only showed the *L. pennellii* specific polymorphism in lines IL 7-2 and IL 7-3 and not in IL 7-1 and IL 7-4. (C) Physical maps of distances between transposed *Ds* elements and their T-DNA on chromosomes 6 and 7 of the tomato plant lines used. At the top, a physical map of *Ds* elements around the D3-5 T-DNA is shown as published before (Stuurman, *et al*., 1998). Position zero indicates the *lox* site in the T-DNA, which is also the approximate donor site for *Ds* transposition. Some plants contained two or more transposed *Ds* elements as indicated in their number. On chromosome 7: plant G3-3-45 contained two *Ds* elements and plant G3-3-44 contained three *Ds* elements as indicated in the map.

targeted and non-targeted transposon tagging as reviewed by May and Martienssen (2003). Secondly, transposed *Ds* elements can be mapped both genetically and physically relative to the D3 T-DNA. Thirdly, this detailed physical map of transposed *Ds* elements around the D3 construct makes it possible to induce chromosomal deletions, inversions or translocations of a chosen size *in vivo* after the introduction of Cre. Integrated use of these three features in plants enables the localization, identification and cloning of genes, as well as the functional characterization of intergenic regions. The strategy is described in more detail by Gidoni *et al*. (2003). This technology could prove especially useful for deleting duplicated genes or larger gene families to yield recessive phenotypes for reverse genetic studies that cannot be obtained using transposon tagging.

Here, we focus on Cre-induced chromosomal deletions to address three aspects of the Cre/*lox* system that have thus far remained underexposed: the attainable size, the germinal transmission of the deletion and the stability of the excised DNA. The first issue is the size of deletions: can Cre induce efficient chromosomal recombination over large genetic distances? To cause recombination, two Cre monomers are bound to the two 13 bp inverted repeats of a single *lox* site before a tetramer complex is formed (Guo *et al*., 1997), in which the two *lox* sites are aligned to enable strand exchange via a Holliday-junction intermediate. It is conceivable that at larger genetic distances, the formation of these tetramer complexes is less likely, thus causing a drop in recombination efficiency. When the Cre/*lox* system is to be used for large chromosomal rearrangements, the efficiency may be determined by the proximity of the *lox* sites. The second issue involves the germinal transmission of chromosomal deletions. Especially during the transmission via the male gamete, haplo-deficiency occurs already when very small deletions are present in the genome (Khush and Rick, 1968). Since most genes are expressed in the male gametes of plants (Tanksley *et al*., 1981), it is likely that deletions that encompass several genes will lead to loss of pollen fitness. Since chromosomal rearrangements can have large effects on fertility in plants (Stebbins, 1958), the results of somatic and germinally transmitted deletions were analysed separately. The third issue is the stability of the deleted double stranded circular DNA in plants: what is the fate of the circle *in planta*? To substantiate claims about transgene removal, using the Cre/*lox* system, it is vital to know the stability of the deleted DNA. Thus far, this aspect of Cre/*lox* recombination *in planta* was only studied in wheat by Srivastava and Ow (2003), where in a rare case a Cre-mediated deletion product was maintained as an extra-chromosomal circular molecule. Therefore they concluded that in this rare case excision products of site-specific recombination may not be subjected to cellular degradation to the

extent that is commonly presumed to occur. However in 68 of the 72 plants Srivastava and Ow studied, normal deletion takes place, which is consistent with our findings.

Experimental procedures

Plant material

The construction of the pD3 construct (Figure 1A) and pMH2626 (the Cre-encoding construct in line C5), as well as their transformation to respectively *L. esculentum* lines UC82B and Moneymaker was described earlier by Coppoolse *et al.* (2003). Single copy lines D3-3 and D3-5 were selected by Southern analysis (not shown).

The location of the D3-5 integration was determined on a molecular linkage map, by RFLP mapping of closely linked *Ds* flanking sequences as described before (Stuurman *et al.*, 1996), using two independent mapping populations. As shown in Figure 1B, D3-5 is located on the long arm of chromosome 6, between markers TG 25 and TG 253. The D3-3 T-DNA is located on the short arm of chromosome 7, in a 10 cM interval between markers TG 199 and TG 217. Crosses to lines carrying the *potato leaf* (*c*), the *mottled*-2 (m2) gene, and *self-pruning* (sp, present in line UC82B) confirmed the position on chromosome 6. Linkage to the *necrotic dwarf*, or *gibberellic acid deficiency*-1 loci was not tested; their approximate map position is derived from Van Wordragen *et al.* (1996).

To map D3-3, the plant DNA flanking the T-DNA was isolated by means of plasmid rescue (Rommens *et al.*, 1992) and mapped on chromosome 7, using a population of 38 *L. esculentum* × *L. pennellii* F2 plants as described before (Stuurman *et al.*, 1996). To confirm the mapping position of D3-3 on chromosome 7, the *Lycopersicon pennellii* introgression lines IL 7-1 to -4 (Eshed and Zamir, 1995) were used. Classical genetic linkage data show linkage of D3-3 to the *notabilis* (*not*) gene (Burbidge *et al.*, 1999), which is present in this same 10 cM interval (Alan Burbidge, personal communication).

To activate the non-autonomous *Dissociator* (*Ds*) element, both D3-3 and D3-5 plants were hand pollinated with s*Ac* line SLJ 10512, containing a non-transposing stabilised Activator (s*Ac*) element encoding transposase and a β-glucoronidase (GUS) reporter gene (Scofield *et al.*, 1992). From the F2 seeds plants with (at least) a single germinally transmitted transposition event were selected and characterised as described by Stuurman *et al.* (1998). These plants were termed Germinals. The *Ds* elements present in the Germinals were mapped, first roughly by classical genetic linkage analysis and later physically by a combination of Pulsed Field Gel Electrophoresis of *Rsr*II fragments and Southern analysis, analogous to Machida *et al.* (1997). The relative orientations of the *lox* sites were determined using the probes and the method described by Stuurman *et al.* (1998).

Germinals with a single *Ds* element were either self-fertilized or out-crossed to determine the genetic distance between the T-DNA (carrying an NPTII gene for kanamycin resistance) and the *Ds* element (carrying a BAR gene for phosphinotricin resistance). Seedlings were sprayed using 0.5 ml/l Finale SL 14 (containing 150 mg/l phosphinotricin, AgrEvo, Holland) and survivors were subsequently sprayed with 400 mg/l kanamycin sulphate (Duchefa, Holland) according to Weide *et al.* (1989). From the relative numbers of phosphinotricin resistant and kanamycin sensitive recombinants, genetic distances were calculated.

To obtain a detailed physical map of the transposed *Ds* elements that were closely linked to the D3 T-DNAs on chromosomes 6 and 7, the procedure of Stuurman *et al.* (1998) was used. Since an *Rsr*II restriction site occurred between the D3-3 T-DNA and the *Ds* element of G3-3-37 at-55 kb on chromosome 7, physical distances between *Ds* elements in lines G3-3-37 and -20 were confirmed by hybridisation of *Ds* flanking sequences to the same 60 kb *Sma*I fragment of YAC V107 D8 of VFNT Cherry on a PFG Southern blot (data not shown).

The physical map with the relative positions and orientations of the *lox* sites shown in Figure 1C was used to plan defined chromosomal deletions by crossing appropriate Germinal lines to a Cre-producing line. Selected Germinals were hand pollinated with pollen of *cre* expressing lines C5, C13, C22 or C43 (Coppoolse *et al.*, 2003). This way, chimaeric F1 plants were obtained with the desired deletion. When the deletion was germinally transmitted to the next generation, stable deletions were obtained in the absence of Cre.

To study somatic deletion activity in tomato, plants G3-3-20, -44, -14, -34, -42 and -45 were fertilized with pollen of *cre* expressing lines C5, C13, C22 or C43. White phenotypes resulting from the deletion of the SppS gene in the F1 plants were photographed using Kodak 5018 EPY films and prepared for publication using Adobe Photoshop 7.0.1 after scanning. Chimaeric plants were allowed to self-pollinate and were reciprocally out-crossed to the wild-type variety 'Moneymaker'. The resulting seeds were both sown in soil and *in vitro* (Coppoolse *et al.*, 2003) to establish the relative numbers of totally white seedlings in each progeny.

Detecting circular excised DNA

From previous experiments (Coppoolse *et al.*, 2003) it was clear that the level of *cre* expression varied with the stage of leaf development, therefore Cre-mediated excision in Germinal × C13 progeny was expected to vary likewise. To isolate comparable DNA samples for the Southern blot in Figure 2B the leaves numbered 6, 9 and 13 (labelled G, J and N) were picked at three different dates (when their over-all length was 10 cm) and stored frozen. Total plant DNA isolation, DNA digestion, electrophoresis and Southern analysis were carried out as described previously (Coppoolse *et al.*, 2003). To detect a circle-specific fragment, the *bar* gene, present on a 633 bp BamHI fragment of pGSFR280 (De Block *et al.*, 1987) was used as a probe. This *bar*-probe included a small part of the *nos* terminator. Circle specific fragments were detected in series of leaves of three different G3-5-60 × C13 progeny plants using the restriction enzymes *Eco*RI, *Rsr*II not shown) and *Sca*I (Figure 2B).

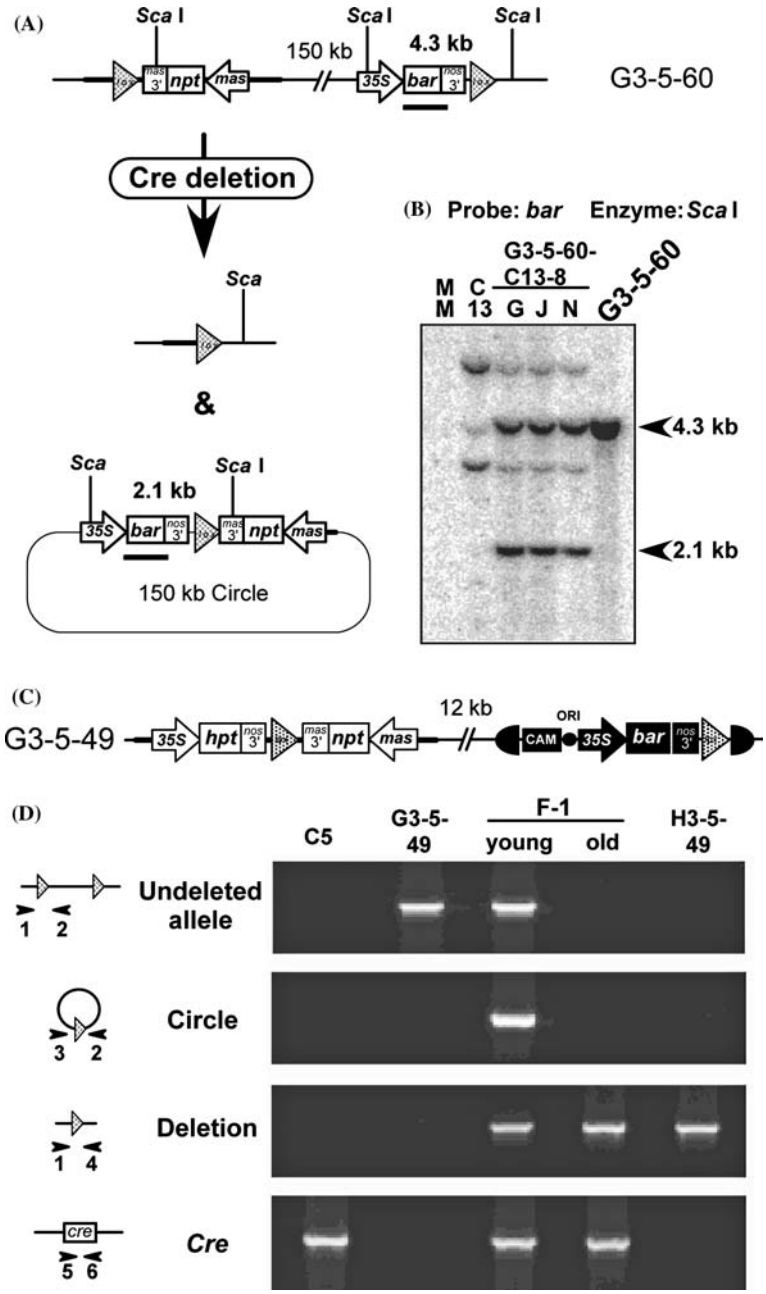
For PCR detection of circular excised dsDNA, the undeleted allele, the deletion and the presence of the *cre* gene, plant DNA was isolated using a scaled-down version of the CTAB method described before (Coppoolse *et al.*, 2003). The plant lines analysed were G3-5-48, -49, -50, -60, -67; G3-3-45, -47, -31, -18, -56, -20, -44, -14, -34, -42 (these lines are represented schematically in Figure 1C) and T933 (containing construct pMV933), which was described before in detail (Coppoolse *et al.*, 2003). The position of the primers used is indicated in Figure 2D, 1 = hptII out: 5′-ACTCGCCGATAGTGGAAACCG-3′, 2 = mas 3′ out: 5′-GCGATATTATTGCCTTTCGCC-3′, 3 = bar out: 5′-CTGCCGGTACCGCCCCGTC-C-3′, 4 = L*Ds* 9: 5′-GGGAATTCCTTGCTCAC-ATCTGGATCAC-3′, 5 = *cre*-top: 5′-ATGTCC-AATTTACTGACCGTA-3′, 6 = *cre*-bot: 5′-TCA-ATCGCCATCTTCCAGCAGGC-3′. As a positive control (for template quality) in PCR assays, part of the aspartate carbamoyltransferase gene of tomato was amplified with the primer set: act F: 5′-GGTTATCTTATGGCTACTCTG-3′ and act R2: 5′-TCCTCCCATCGAACCCC-3′. All primer combinations were used in the same regime: 60 s T = 94 °C, 45 s T = 59 °C, 60 s T = 72 °C, repeated for 35 cycles in a 50 μl volume, using a DNA Thermal Cycler (Perkin Elmer, USA).

For plasmid rescue of circular excised dsDNA, total plant DNA was extracted from 15 pooled T933 × C5 seedlings using the CTAB protocol described before (Coppoolse *et al.*, 2003). The DNA was dialysed against distilled water on a micropore nitrocellulose filter for an hour and 1 μg was used for a single electro-transformation of competent NM554 cells (Raleigh *et al.*, 1988) at 20 kV/cm using a Gene Pulser machine (Bio-Rad). The transformation mixture was plated on LB medium containing 35 mg/l chloramphenicol and the plasmid content of resistant clones was characterized by restriction analysis.

Results

Induction of somatic deletions

A series of deletions was made on chromosome 7 by crossing homozygous lines G3-3-20, -44, -14, -34, -42 and -45 to the homozygous Cre lines C5, C13, C22 or C43 (Table 2). These deletions all encompassed the Solanesyl diphosphate Synthase (SppS), previously referred to as a putative prenyl transferase gene (Coppoolse *et al.* 2003). The SppS gene is involved in chlorophyll biosynthesis (data not shown) and deletion causes a dominant white phenotype in all F1 progeny plants. The visible phenotypes in F1 plants that result from the crosses with C5 are shown in Figure 3; For Moneymaker, G3-3-20, -44 and -14, a leaf is shown that is representative for the two or three F1 plants that were grown to maturity (Table 2), while for lines G3-3-34 and G3-3-45 the largest of a few sectors is shown. Different gradations of the white phenotype were observed; in F1 progeny



plants of G3-3-20 × C5 (deletion of 7.5 kb of plant DNA), the leaves became completely white, whereas in the three F1 plants of G3-3-34 × C5 (deletion of 65 kb of plant DNA) only a few white sectors were formed (Figure 3).

In plant G3-3-44, three *Ds* elements were mapped (coined 44-1 to -3), shown as grey triangles in Figure 3. Crossing these lines to the *cre* expressing line C5 gave rise to large white sectors throughout the plant, comparable to G3-3-14 when crossed to C5. Presumably, the efficiency of deleting the SppS gene was determined by the *Ds* element that caused the smallest deletion that includes the SppS gene, coined 44-2 in Figure 3.

Figure 2. (A) Schematic representation of the essential elements in G3-5-60 (not to scale, for details, see Stuurman, *et al*. 1998). Plant G3-5-60 harbours two *lox* sites (indicated by grey triangles), which are in direct repeat, bracketing a mas 5′::nptII::mas 3′ cassette and a 35S::bar::nos 3′ cassette, separated by a stretch of plant DNA of 150 kb. The positions of relevant *Sca*I sites and the size of the expected fragments after digestion are indicated. The approximate size and the position of the *bar* probe used for the Southern blot shown in panel B is indicated as a horizontal black bar. (B) Autoradiograph of a Southern blot after hybridisation with the *bar* probe. From left to right, lanes contain *Sca*I digests of: Money Maker (MM): the untransformed control, parent C13, the DNA of three leaves of F1 plant G3-5-60C13-8: G (leaf number six), J (leaf number nine), N (leaf number 13) and parent G3-5-60. From the undeleted allele, a 4.3 kb fragment is labelled, after deletion the probe hybridises to a 2.1 kb circle-specific fragment. The additional bands that are also visible in lane C13 are caused by the small *nos3′* fragment in the *bar* probe that hybridised with the three copies of the pMH2626 construct in line C13 (Coppoolse *et al*., 2003). (C) Schematic representation of the essential elements in G3-5-49 (not to scale, for details, see Stuurman, *et al*., 1998). Plants G3-5-49 harbours two *lox* sites (indicated by grey triangles), which are in direct repeat, bracketing a mas 5′::nptII::mas 3′ cassette and a 35S::bar::nos 3′ cassette, separated by a stretch of plant DNA of 12 kb. (D) PCR fragments in an ethidium bromide stained agarose gel. The positions of the primer sets used to PCR amplify the DNA fragments shown in each of the 4 panels are indicated schematically on the right. The template DNAs are indicated at the top of the four panels, from left to right the C5 and G3-5-49 parents respectively, the next two lanes are obtained from the same F1 plant, 'young' indicates DNA isolated from the primary leaf, 'old' represents DNA of leaf number 20. In the last lane, DNA of line H3-5-49 is used. This line resulted from the 12 kb Cre-induced deletion in G3-5-49 and serves as a positive control for the deletion allele.

We assume that inversions between the *lox*-sites named 44-3 on one end and 44-2, 44-1 and the *lox*-site in the T-DNA on the other end occurs until *lox*-site 44-3 is deleted. Possibly, deletion of the 41 kb between 44-2 and 44-1 is also efficient, leading to the somatic deletion of 96 kb, which is not germinally transmitted (Table 2).

In F1 plants of G3-3-45 crossed to *cre* expressing line C5 a deletion of about 200 kb was expected that contained the SppS gene. Somatic deletion of 200 kb appeared to be a rare event. As shown in Figure 3, only a few greyish coloured deformations along the leaf edges of some older leaves were observed. Clearly deletions occur less efficient when the size of the deletion increases and probably at this locus, 200 kb is the limit to the size of somatic deletions that can be induced with Cre line C5.

Induction and inheritance of germinal deletions

The transmission of the deletions on chromosome 7, including the SppS gene, was analysed in selfings and reciprocal out-crossed progeny of the chimaeric plants that are shown in Figure 3. White seedlings, lacking all chlorophyll, where expected in the progeny of all crosses because of the dominant character of the phenotype. It was found that deletions were not transmitted via the male gametophyte. White seedlings were only observed after the chimaeric F1 plant was self fertilised or pollinated by the wild type 'Money maker' plant. No white seedlings were observed in progeny of the reciprocal cross. The germinal transmission frequency (GTF) after selfing is calculated (in Table 2) as the number of totally white seedlings (W) per total number of seedlings (T), divided by the theoretical maximum percentage white seedlings (50%) that can be obtained: GTF = (W/T) / 50%. In F2 progenies of G3-3-20 and G3-3-14 crossed to *cre* expressing line C5, germinal transmission of respectively the 8 kb and the 55 kb deletions occurred with an average GTF of respectively 83% and 5.2%. Apparently, deletions can be transmitted via the female gametophyte, but the efficiency is lower than might be expected on the basis of the prevalent occurrence of somatic phenotypes (Figure 3).

The stability of circular Cre-mediated deletion products in planta

The action of Cre is to circularise *lox*-embedded DNA. There is no apparent reason to question the circularising mechanism in plant cells, but it is not clear how stable a dsDNA circular molecule is in plant cells. In three experiments we show in somatic cells the abundance of the deletion circle, the intact circular form and the stability of the circle after its formation.

To show the abundance of a somatic 150 kb dsDNA deletion-product of line G3-5-60 (described by Stuurman *et al*., 1998), the *bar* gene was used as a probe to detect a circle-specific 2.1 kb *Sca*I fragment on a Southern blot (Figure 2B). Similar results were obtained using series of leaves of two other G3-5-60 × C13 progeny plants using the restriction enzymes *Eco*RI, *Rsr*II (not shown). This somatic deletion was apparently induced in maturing leaves throughout the F1

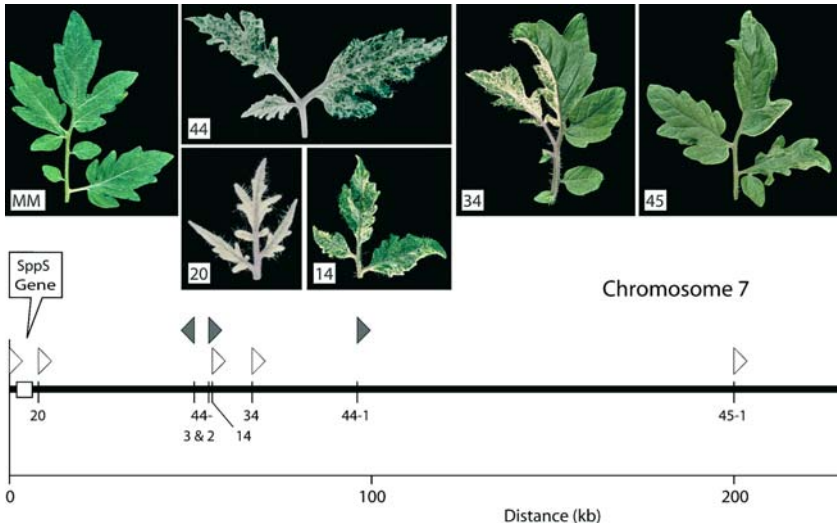


Figure 3. Increasing deletion size correlates with decreasing Cre-mediated deletion efficiency. At the bottom of the panel, a schematic map is drawn, showing the orientation of the *lox* sites in the *Ds* elements of the G3-3 plants and their corresponding numbers. The distance over which the *Ds* elements transposed from the D3-3 T-DNA (at position 0) is indicated in kb. The T-DNA inserted next to a Solanesyl diphosphate Synthase (SppS) gene and its deletion results in a dominant white phenotype. This white phenotype is used as a visual marker to compare relative deletion efficiencies between tomato plants with deletions of increasing size, as shown in the top panels. For Moneymaker, G3-3-20, −44 and -14, a leaf is shown that is representative for the F1 plants studied, while for lines G3-3-34 and G3-3-45 the largest of a few sectors is shown. In G3-3-20 an 8 kb deletion occurs with 100% efficiency in secondary leaves, which become completely white, while larger deletions show less white sectors, indicating lower deletion efficiencies.

plant by crossing to Cre line C13. In earlier experiments (Coppoolse *et al*., 2003) the expression of the *cre* gene in line C13 was shown to occur relatively late in leaf development, which explains the abundance of the 2.1 kb band in Figure 2B.

To prove that Cre-mediated deletion results in an intact circular molecule, total DNA of T933 × C5 seedlings was used to transform electro-competent *Escherichia coli* cells. The C5 line expresses *cre* early after seed germination and in F1 seedlings of a T933 × C5 cross the deletion is completed before the secondary leaves are formed (Coppoolse *et al*., 2003). The circular deletion product of T933 contains a bacterial origin of replication and a bacterial chloramphenicol resistance gene. After transformation the *E. coli* cells indeed contained the expected 8.4 kb plasmid.

To assess the stability of Cre-mediated dsDNA deletion-circles *in planta*, F1 seedlings of 25 crosses were analysed with PCR as listed in Table 1. For illustration, a typical PCR result is shown in Figure 2D. In the total of 189 plants where Cre-mediated deletion took place, in only 90 cases the circle was detected by PCR. In all 90 cases, the undeleted allele was also detected. In older plants, not only the undeleted allele was gone, also the circle-specific PCR product could no longer be amplified. Although the number of plants in which the circular DNA was actually detected is limited, this suggests that circular dsDNA, ranging in size from 5 kb up to 220 kb, is not stably maintained in tomato.

Discussion

What may be the fate of circular Cre-deletion products in planta?

The double stranded DNA circles produced by Cre-mediated deletion are instable in tomato. A PCR-product spanning the *lox* site on the deletion circle can only be amplified in plants that still contain some of the non-circularised DNA. The fact that no *lox* site spanning PCR-product can be amplified after the deletion is completed, shows that this DNA is probably not randomly inserted elsewhere in the genome. Cre-mediated reintegration at a (pseudo) *lox*-site elsewhere in the genome has been studied thoroughly in *Arabisopsis thaliana* (Vergunst and Hooykaas, 1999) and the chance

Table 1. PCR analysis of somatic Cre-mediated deletion in F1 plants.

Cross		Seedlings analyzed	Cre 5&6	Undeleted 1&2	Circle 2&3	Deletion 1&4
Lox line	Cre line					
G3-5-48	C5	5	2	2	1	1
G3-5-49	C5	26	22	21	5	16
G3-5-50	C5	23	15	15	10	12
G3-5-67	C5	23	19	4	2	19
G3-5-60	C13	4	4	1	1	1
G3-3-20	C5	2	2	2	2	2
	C13	38	30	26	25	25
	C43	7	7	5	5	5
G3-3-14	C5	3	2	2	2	2
	C13	5	5	3	1	2
G3-3-34	C5	4	4	4	4	4
	C43	6	6	5	4	4
G3-3-42	C5	3	3	3	3	0
	C13	3	3	3	2	1
G3-3-56	C5	2	2	1	1	1
	C22	3	3	2	1	1
G3-3-18	C5	3	3	3	3	3
	C13	4	4	4	3	4
	C22	2	1	1	1	1
G3-3-31	C5	2	2	1	1	1
	C13	2	2	1	1	1
	C22	2	2	2	1	1
G3-3-47	C5	4	4	4	4	4
	C22	4	4	3	1	1
	C43	9	8	6	6	6
Total		189	159	124	90	118

To analyse the efficiency of Cre-mediated somatic deletion in tomato chromosomes 6 and 7, the (homozygous) *lox*-sites containing lines that are schematically shown in Figure 1C were crossed to the homozygous Cre lines as indicated. The quality of the DNA that was isolated from the seedlings was checked with Cre primers (primers 5 and 6, see text), before primer sets 1&2, 2&3 or 1&4 were used. The numbers in the table indicate the numbers of plants that gave a PCR product with the indicated PCR primers. In all cases where a circle-specific fragment could be amplified, the undeleted allele was also detected.

that this occurs is minute. Therefore it is likely that the deleted DNA is lost, presumably through endonuclease activity in the tomato cell or, when somatic deletion is complete in an early stage of plant development, by dilution as a result of later cell divisions. These data are consistent with the results of Srivastava and Ow (2003), who could detect a deletion circle in wheat plants containing both a particular *cre* locus (*cre37*) and the complex pVS11 locus. In their Southern blots, the deletion circle is always accompanied by the undeleted allele, which suggests that the deletion circle is most likely generated *de novo*. In contrast to the suggestion of Srivastava and Ow (2003) that the deleted DNA remains in the cells as an extra-chromosomal circular molecule, we conclude that their results as well as ours support the hypothesis that the deletion circle is instable and soon lost after its conception.

Why do larger somatic deletions occur less frequently than smaller ones?

For the induction of small deletions in somatic cells, the Cre/*lox* system can be very efficient (Coppoolse *et al.*, 2003). However, the results presented in Figure 3 show that the efficiency of somatic deletion drops when the size of the deletion increases. Size dependency was also observed in the *Drosophila* genome by Golic and

Table 2. Numbers of chimaeric and totally white seedlings in F1 and F2 progeny of G3-3 deletion lines.

Cross		Numbers of F1 seedlings				Numbers of F2 seedlings			
lox line	Cre line	Total	Chimaeric	Green	Grown to maturity	Total	White	Green	GTF (%)
G3-3-20	C5	5	5	0	1*	29	12	17	83
	C13	38	30	8	5	690	156	534	45
	C22	6	3	3	2	99	20	79	40
	C43	9	2	7	2	98	0	98	0
G3-3-44	C5	7	4	3	3	98	0	98	0
	C13	7	3	4	2	198	0	198	0
G3-3-14	C5	8	3	5	2	77	2	75	5.2
	C13	6	3	3	2	42	0	42	0
G3-3-34	C5	7	5	2	3	77	0	75	0
	C13	7	0	7	2	197	0	197	0
	C43	6	0	6	1	21	0	21	0
G3-3-42	C5	11	nd	nd	0	nd	nd	nd	nd
	C13	7	0	7	2	198	0	198	0
G3-3-45	C5	12	1	11	3	98	0	98	0
	C22	8	0	8	2	99	0	99	0

To quantify the comparison between the somatic deletion efficiency and the germinal transmission frequency of Cre mediated deletions on chromosome 7, the numbers of visibly chimaeric F1 plants (at a plant-length of roughly 15 cm) are listed next to the calculation of the Germinal Transmission Frequency (GTF) for the same cross. The listed numbers of green seedlings include the seedlings with the Cre phenotypes that were described before (Coppoolse, *et al*., 2003). From the five G3-3-20 × C5 plants, only one (*) made a side-branch containing enough chlorophyll to bear some fruits. The progeny of the G3-3-42 × C5 cross was infected by a virus and was discarded after PCR analysis (Table 1) was finished; numbers of chimaeric F1 seedlings and white F2 seedlings could therefore not be determined (nd). The Germinal Transmission Frequency is used here as a measure for Cre efficiency and is calculated as GTF = (W/T)/50%, in which W is the number of white seedlings and T is the total number of seedlings. Since deletions that include the SppS gene are not transmitted via the pollen (Coppoolse, *et al*., 2003), a fraction W/T of 0.5 results in a GTF of 100%.

Golic (1996), who used the FLP/*FRT* site-specific recombination system to induce chromosomal rearrangements. Presumably, the probability to align two recombinase recognition-sites in a recombination complex decreases when these sites are further apart. On chromosome 6 of tomato however, the size limitation to align two *lox*-sites in G3-5-60 seems to become only relevant for distances exceeding roughly 150 kb (Figure 1C), since somatic Cre-induced deletions up to this size are induced efficiently (Figure 2B). Somatic deletions on chromosome 7 though, are rare over distances of 60 kb and more, which may suggest selection against deletion events. Dependent on the information content of the deleted DNA, deletion may cause physiological adverse effects. However, no signs of selection against cells carrying such deletions were observed: the large green areas of chimaeric plants G3-3-34 and G3-3-45 appeared completely normal (Figure 3). Therefore we reject the idea that there is effective selection against large somatic deletions and we assume that the induction-frequency of larger somatic deletions is lower compared to the induction-frequency of smaller somatic deletions on the same locus.

Germinal versus somatic deletions

Medberry *et al*. (1995) produced a somatic Cre-mediated deletion event in tobacco. Based on genetic mapping, this deletion was expected to be large and it was not transmitted to the next generation. Especially for the larger Cre-mediated deletions, we observed a similar pattern in tomato. The differences in frequency of occurrence between somatic and germinal deletions may be due to different reasons. First, deletions can cause chromosomal instability. However, from a cytological point of view, instability as a result of these relatively small deletions is unlikely. Second, the activity of Cre in germline cells may be too low. Differences in the expression levels of the *cre* gene in germ line cells compared to somatic cells may occur. However, deletions in various tester constructs were transmitted very efficiently through meiosis (Coppoolse *et al*., 2003). Obviously, poor *cre* expression in germ line cells cannot be the (only) reason for low or absent transmission of rearrangements via the germ line. Third, Cre toxicity causes somatic phenotypes in tomato (Coppoolse *et al*., 2003), which may pose a

problem in gametes especially when expressed to high levels as shown in mammalian cells (Schmidt *et al.* (2000); Loonstra *et al.* (2001)). Selection against Cre containing gametes can explain low over-all transmission frequencies of deletions through the germ line, but Cre toxicity is not expected to differentiate between larger and smaller deletions. Fourth, deletions may affect gametophyte fitness. In the larger deletions, genes may be lost that are necessary for proper gametophyte performance. According to Van der Hoeven *et al.* (2002), one gene is expected on average in a 10 kb sequence of a euchromatic region. Tanksley *et al.* (1981) concluded that about 60% of the sporophytic gene repertoire of tomato is also used in the gametophyte. Honys and Twell (2003) estimate the overlap in gene repertoire at 61% in *Arabidopsis thaliana* and they claim that nearly 40% of all genes are pollen specific. Selection against germinal transmission of larger chromosomal deletions is likely to be caused by fitness selection in the gametophyte. The data put forward in this study support the notion that for somatic Cre-mediated deletion and the germinal transmission of deletion events, size does matter.

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