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Strategies for targeted transposon tagging of ABA biosynthetic mutants in tomato

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Abstract The ABA biosynthetic pathway has been studied in detail and the steps impaired in some ABA-deficient mutants are known. However, little is known of the molecular control mechanisms regulating ABA production in planta. A direct route for improving our understanding of these mechanisms is to transposon tag and clone the wild-type counterparts of the ABA mutant alleles. On the basis of the observation that maize transposons move preferentially to linked sites in both homologous and heterologous systems and in doing so disrupt gene function, a targeted transposon mutagenesis strategy is being developed towards cloning ABA biosynthetic genes from tomato. The possibility of using marker genes to identify T-DNA insertion sites in selected parts of the genome has been examined and compared with an inverse PCR/RFLP approach to mapping T-DNAs.

Key words Tomato · *Agrobacterium* T-DNA · Inverse PCR · ABA · Transposon tagging

Introduction

The ABA biosynthetic pathway has been extensively studied and the precise steps affected in some ABA deficient mutants have been established (Sindhu and Walton 1988; Taylor et al. 1988; Parry et al. 1988; Duckham et al. 1991; Rock and Zeevaart 1991). These mutants are characterised by the fact that under conditions of water stress they wilt much more rapidly and more 'dramatically' than normal plants (Tal and Nevo 1973). Although the biosynthetic pathway is now fairly well-characterised (Zeevaart and Creelman 1988; Taylor 1991), the enzymes catalysing each reaction have not yet been isolated and the control systems employed in planta regulating ABA concentration remain poorly understood.

There are three ABA deficient mutants of tomato and the chromosomal locations of these mutant genes are known (Stevens and Rick 1986; Mutschler et al. 1987). The mutation, *sitiens* (*sit*), is located on the short arm of chromosome 1 (Fig. 1); the two remaining mutants are located on the long arm of chromosome 7 (Fig. 1). Two of the mutants, *flacca* (*flc*) and *sit*, affect the same step in the pathway i.e. the oxidation of ABA aldehyde to ABA (Sindhu and Walton 1988; Taylor et al. 1988). The other mutant, *notabilis* (*not*), appears to be unable to carry out the normal oxidative cleavage reaction in the middle of the pathway that releases the first C_{15} intermediate, xanthoxin, from the final C_{40} precursor 9′-*cis*-neoxanthin (Taylor 1991; Parry et al. 1992).

One route towards isolating these genes, other than by biochemically based studies, is the use of transposon tagging. Maize transposons have been shown to move preferentially to sites genetically linked to the donor site (Van Shaik and Brink 1959; Dooner and Belachew 1989) and this attribute is maintained in heterologous systems (Dooner et al. 1991; Thomas et al. 1994). These properties are exhibited by the maize *Activator* (*Ac*) transposon and can be exploited in a targeted transposon tagging strategy to clone ABA biosynthetic genes from tomato.

The three ABA-deficient mutants are difficult to identify individually if they are segregating in the same population. Alternative mutant marker loci have been placed on the classical map of *L. esculentum* to positions very close to each of the ABA deficient mutant gene loci (see Fig. 1). The phytochrome-deficient mutant *aurea* (*au*) maps to the same position as *sit*, i.e. at map position 32 cM on chromosome 1 (Stevens and Rick 1986). On chromosome 7, the sideshootless mutant *lateral sup-*

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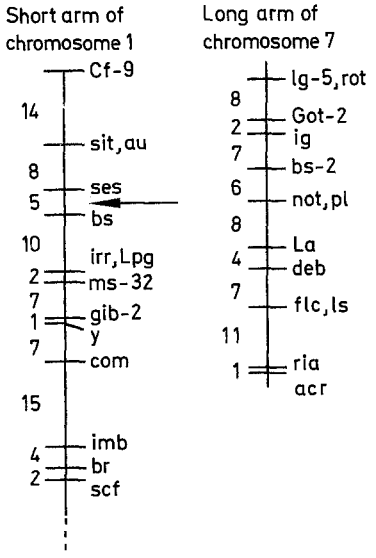


Fig. 1 Classical maps of the regions of chromosome 1 and 7 in which the ABA mutant genes are located. The *arrow* on chromosome 1 indicates the approximate position of T103 T-DNA (see Table 1). Chromosome 1 data are from Balint-Kurti et al. (1994); chromosome 7 data from Mutschler et al. (1987)

pressor (*Is*) is at the same site as *flc* (map position 59 cM), while *perlucida* (*pl*), a mutant affecting leaf shape and colour, maps to the same location as *not* i.e. map position 40 cM (Stevens and Rick 1986).

This paper demonstrates the effective use of these marker loci to identify transformants carrying T-DNA/*Ac* insertions in preselected regions of the tomato genome. This approach does not require an extensive knowledge of the organisation of a species' genome and yet allows putative transformants to be selected that have T-DNAs/transposons in positions linked to specified genes. This method has been compared with a restriction fragment length polymorphism (RFLP) mapping approach, based on inverse polymerase chain reaction (IPCR), where transformants were generated at random and the position of their T-DNA established subsequently.

Materials and methods

Generation of the triple mutant marker genotype (*au au, pl pl, Is Is*)

Recessive alleles of the three gene loci co-mapping with the ABA mutants (*au, pl, ls*, Stevens and Rick 1986) were combined into one line (homozygous triple mutant) by selecting recombinants from crosses between homozygous single and double mutant lines. The resulting homozygous triple mutant was used to pollinate the variety 'Ailsa craig' to produce the F_1 heterozygous triple mutant (*au* +, *pl* +, *ls* +).

Generation of transformants

Cotyledons of the F_1 heterozygous triple mutant, grown in vitro, were transformed according to the method of Fillatti et al. (1987) using *Agrobacterium tumefaciens* with the T-DNA::*Ac* construct SLJ591 (Jones et al. 1992; Thomas et al. 1994). This construct was donated by Dr. J. Jones, Sainsbury Lab, Norwich, UK (see Fig. 2). Putative hemizygous, transformant cotyledon explants were subcultured monthly onto modified Murashige and Skoog medium [containing 0.7% (w/v) agar, 3% (w/v) sucrose, 1 mg l^{-1} thiamine-HCl, 0.5 mg l^{-1} nicotinic acid and 0.5 mg l^{-1} pyridoxine] supplemented with 10 μM zeatin, 500 mg l^{-1} carbenicillin and 100 mg l^{-1} kanamycin. These explants were maintained in a growth room at 25 °C with cool white light for 16 h day^{-1}. Regenerated tomato plantlets were rooted on the modified Murashige and Skoog medium (as above) supplemented with 200 mg l^{-1} carbenicillin and 30 mg l^{-1} kanamycin. The resulting putatively transformed tomato plants were transferred to a glasshouse. Daylight was supplemented when necessary to give a 16 h day and 8 h night regime. The minimum day temperature was 25 °C and the minimum night temperature was 18 °C. Plants were grown in Levington M3 compost (Fisons, Loughborough, UK).

Classical linkage analysis of transformants

Putative primary transformants were grown to the second flower truss. They were allowed to self at the first truss and at the second truss were backcrossed to the homozygous triple mutant (*au au, pl pl, ls ls*). Small backcross populations of about 500–1000 seeds from each putative, primary transformant were generated. A sample of about 200–400 seedlings from each of these populations was sprayed on 3 consecutive days with an aqueous solution of kanamycin (400 mg l^{-1}) after the method of Weide et al. (1989). Plants carrying the SLJ591 T-DNA had resistance to this antibiotic conferred by the transformation marker NPTII (Fig. 2). Susceptible plants developed chlorotic lesions 5–7 days after the kanamycin treatment. The backcross populations were subjected to segregation analysis. The three mutant marker gene phenotypes were scored, and evidence of linkage between any of them and the T-DNA was assessed.

T-DNA copy number and *Ac* excision

Small F_2 populations (about 60 seeds), from each putative, primary transformant were germinated in 9-cm petri dishes on filter paper discs saturated with aqueous spectinomycin solution (100 mg l^{-1}). Seedlings susceptible to this antibiotic had purple (anthocyanin-rich) hypocotyls and bleached cotyledons; resistant seedlings developed normally. Populations that segreated 3:1 for antiobiotic resistance conferred by this dominant, non-cell autonomous excision marker (Fig. 2) were likely to harbour one T-DNA::*Ac* copy with an actively excising *Ac* element. Populations that segregated 15 resistant to 1 susceptible were presumed to have two T-DNA::*Ac* copies with actively excising *Ac* elements.

Ploidy of transformants

Leaf tissue was collected from each primary transformant. Lower epidermal strips were examined under 40 × magnification. The number of chloroplasts in each of ten pairs of stomatal guard cells were counted and averaged. The ploidy level of each transformant was estimated by counting the number of chloroplasts in guard cells according to the method described by Koornneef et al. (1989). Putative diploid transformants which, on the basis of the above spectinomycin test appeared to have one T-DNA copy with an actively excising *Ac* element, were selected for classical linkage analysis and preferentially used for inverse PCR.

DNA isolation for inverse PCR

Backcross or F_2 populations were sprayed with kanamycin as described earlier to select individuals carrying the T-DNA. Tomato genomic DNA was isolated according to the methodology detailed in Thomas et al. (1994) except that 5-ml glass homogenisers (Jencons, UK) were used to disrupt the tissue.

Inverse PCR reactions

Approximately 2 μg of transformant DNA, prepared as above, was digested with *Bst*Y1. Half of the product was redigested with *Bcl*1 (Thomas et al. 1994). Restriction digests were carried out according to the manufacturer's protocols (New England Biolabs). The enzyme *Bst*YI cleaves *Bam*HI, *Bgl*II and *Bam*HI/*Bgl*II fusions to leave 5'GATC cohesive termini. The enzyme *Bcl*I cleaves TGATCA and also creates 5'GATC cohesive termini. There are *Bam*HI cleavage sequences located just within both the left and right borders of the T-DNA. Primer sequences located external to these *Bam*HI sites were used in inverse PCR reactions (Fig. 2). Following phenol:chloroform extraction and ethanol precipitation, digested DNA samples were circularised at a concentration ≤ 2.5 μg ml^{-1} in the presence of 10 U ml^{-1} T4 DNA ligase (BRL) at 12 °C for 16 h. Ammonium acetate was added to 1 *M* and the ligation mixture phenol:chloroform extracted and ethanol precipitated (Sambrook et al. 1989). Inverse PCR reactions were assembled as described in Thomas et al. (1994). Primers identical to those used by Thomas et al. (1994) were used for the amplification of tomato genomic DNA flanking left and right T-DNA borders (see Fig. 2). The circularised DNA samples were subjected to 35 cycles using a Techne PHC-3 thermocycler of the following PCR regime: 15 s at 95 °C, 15 s at 55 °C and 60 s at 72 °C. The reaction was completed after a 10-min incubation at 72 °C. Inverse PCR products were resolved in 2% (w/v) composite agarose gels (3 parts Nusieve GTG agarose: 1 part FMC SeaKem agarose) in 1 × TBE buffer (Sambrook et al. 1989). Products from these primary inverse PCR reactions were diluted 100-fold and 5 μl of this mixture was used in a second PCR reaction using a set of 'nested' primers internal to the first pair (see Fig. 2). The concentration of these primers was 10-fold lower than those used in the primary reaction. The reaction volume was 100 μl and the PCR profile was identical to that used in the primary reaction.

Cloning inverse PCR products

Reaction mixtures from nested inverse PCR reactions were extracted with phenol:chloroform. DNA was resuspended in 50 μl of T4 DNA buffer:66 m*M* Tris-HCl pH 8.8, 7 m*M* $MgCl_2$, 1 m*M* β-mercaptoethanol, 17 m*M* $(NH_4)_2SO_4$, 3 m*M* Na_2EDTA, 2 μ*M* dNTPs, 0.05 μg BSA, 1 U T4 DNA polymerase (NEB). This mixture was incubated at 37 °C for 45 min. The resulting 'polished' fragments were extracted with phenol:chloroform and ethanol precipitated. DNA was resuspended in 10 μl distilled water and electrophoresed in 2% (w/v) composite agarose gels. The appropriate fragments were excised from the gel and the DNA isolated using 'Geneclean' (Bio 101). DNA was ligated into the pUC 8-based vector pKRV (Waye et al. 1985) and linearised by digestion with *Eco*RV. Ligated DNA was used to transform *E. coli* JM83 and recombinant clones were identified by the *lac* complementation assay. Authenticity of recombinant clones was verified by digesting alkali lysis-prepared plasmid with *Eco*RI and *Hin*dIII to release the PCR fragment. Sequencing of the PCR fragments' termini (see below) was carried out as a further check of authenticity.

DNA sequence analysis

Plasmid DNA was prepared from 5 ml of a stationary phase culture by alkaline lysis. Alkali-denatured DNA was used as a template in chain-terminating DNA sequencing reactions (Sambrook et al. 1989) and analysed in 6% (w/v) polyacrylamide/8 *M* urea gels.

DNA isolation for Southern analysis

Total DNA was extracted using a method developed by S. Tanksley's group (Cornell University, Ithaca, N.Y.), with some minor modifications. Between 2 g and 3 g of recently expanded tomato leaf tissue was harvested and stored on ice. The tissue was homogenised under liquid nitrogen using a pestle and mortar. The powder was mixed with 20 ml ice-cold extraction buffer (0.35 *M* sorbitol, 0.1 *M* Tris-HCl pH 7.5, 5 m*M* Na_2 EDTA. Sodium bisulphite was added to 36 m*M* just prior to use). The homogenate was vortexed and centrifuged at 2000 g for 20 min. The supernatant was discarded, the pellet resuspended in 2.5 ml ice-cold extraction buffer, 3.5 ml nuclear lysis buffer (0.2 *M* Tris-HCl pH 8, 50 m*M* Na_2EDTA pH 8, 2 *M* NaCl, 50 m*M* CTAB), and 1.2 ml 5% (w/v) sodium lauryl sarcosine were added. The solutions were gently mixed by inversion and incubated at 65 °C for 30 min. The homogenate was extracted with phenol:chloroform and the layers clarified by centrifugation at 2000 g for 20 min. DNA from the aqueous upper phase was precipitated with an equal volume of isopropanol and spooled out into a 1.5-ml microcentrifuge tube. The DNA was rinsed with 80% aqueous ethanol and resuspended in 400 μl sterile distilled water. Routinely, 15 μl of this DNA was sufficient to give a clear signal on a genomic Southern.

Southern hybridisation analysis

DNA samples for Southern analysis were resolved on 1% agarose (SeaKem, FMC) in 1 × TBE buffer (Sambrook et al. 1989). Electrophoresed DNA was depurinated in 0.25 *M* HCl for 15 min, denatured for 45 min in 0.5 *M* NaOH, 1.5 *M* NaCl and neutralised in two 30-min washes of 0.5 *M* Tris-HCl pH 7.5, 1.5 *M* NaCl. DNA was then capillary-blotted for 16 h in 10 × SSC onto Zetaprobe GT nylon membrane (BioRad) and fixed by UV crosslinking (12 000 μJ). Membranes were hybridised for 16 h in glass tubes (Techne) at 65 °C in 15 ml hybridisation solution (5 × SSPE, 5 × Denhardt's 1%SDS, 200 μg ml^{-1} denatured salmon testes DNA; Sambrook et al. 1989). Filters were washed at 65 °C for 30 min in 1% (w/v) SDS, 2 × SSC and for 1 h at 65 °C in 1%(w/v) SDS, 0.2 × SSC.

Preparation and radio-labelling of inverse PCR fragments

Between 10–30 ng of alkali-denatured plasmid DNA containing the required inverse PCR fragment was subjected to 35 cycles of PCR, as described above, in the presence of 1 μ*M* M13 forward and reverse primers. The amplified fragment was resolved in 2% composite agarose (3 parts Nusieve, 1 part FMC SeaKem) and purified using 'Geneclean' (Bio101). Approximately 25 ng of the fragment was labelled with α-[^{32}P]dCTP (110 TBq/mmol) using an 'Oligo-labelling' kit (Pharmacia).

Molecular mapping

Labelled inverse PCR fragments were used to probe filters of *L. esculentum* and *L. pennellii* DNA digested with *Dra*I, *Eco*RI, *Eco*RV and *Hin*dIII to generate an RFLP. Once the enzyme that produced the RFLP was identified, DNA from a population of 34 F_2 plants from a cross between *L. esculentum* and *L. pennellii*, generated and kindly supplied by Dr. S. Tanksley (Cornell, USA), was digested with this enzyme and probed with the inverse PCR fragment. The molecular map position of each T-DNA was determined using the computer programme 'Mapmaker' (Lander et al. 1987) in conjunction with data for the segregation, in the population of 34 F_2 plants, of over 60 single-copy sequences covering the 12 tomato chromosomes (Tanksley et al. 1992).

Results

Generation of transformants

In excess of 200 primary tomato transformants were generated by transforming over 10 000 cotyledons of a heterozygous triple mutant genotype (*au* +, *ls* +, *pl* +) using the wild-type *Ac* carrying T-DNA construct SLJ 591. This construct is known to confer kanamycin (NPTII) resistance onto transformants and spec-

tinomycin resistance (SPEC) upon excision of the *Ac* element (Fig. 2). Only transformants believed to be diploid were subjected to further analyses. Of the total number of putative transformants that were hardened off into glasshouse conditions, approximately 21% appeared to be tetraploids. Diploid individuals were typically allowed to self-pollinate at the first inflorescence and were backcrossed at the second inflorescence with pollen from the triple mutant homozygote (*au, au, ls, ls, pl, pl*).

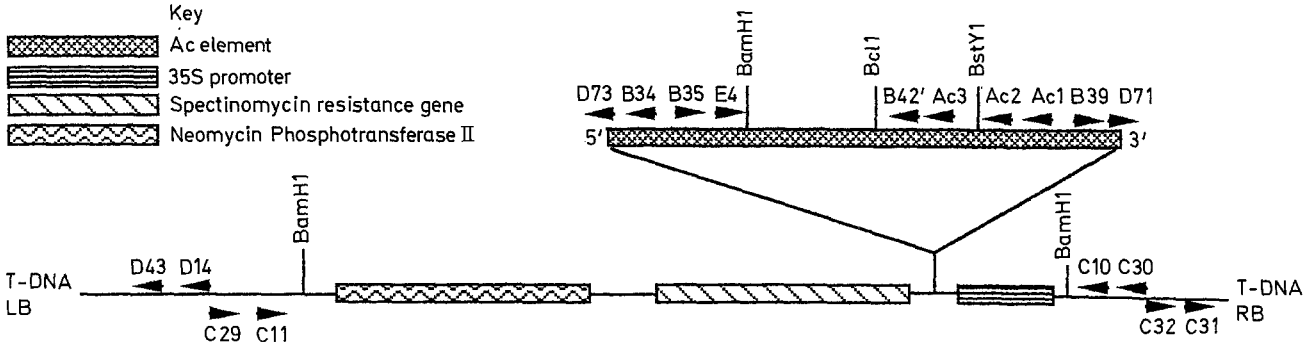
Diploid transformant F_2 populations were routinely germinated on filter paper discs saturated with spectinomycin solution, as a preliminary method of selecting the best candidates for further analysis. The excision marker, although non-cell autonomous, gave an indication that *Ac* was active in at least some cells of the selected transformant lines as well as an indication of the probable number of T-DNA insertion sites. Of an initial sample of putative diploid transformants that had produced sufficient selfed seed for screening, 49% gave a 3:1 ratio of spectinomycin resistant:susceptible seedlings, indicating the presence of one active T-DNA::*Ac* element; 7% gave approximately a 1:1 ratio, perhaps due to lower *Ac* activity in some plants; and 9% gave a 15:1 ratio of spectinomycin resistant:susceptible seedlings, suggesting the presence of two independently segregating active T-DNA::*Ac* elements in these individuals. In the remaining lines (35%) it was likely that the *Ac* element was relatively inactive, producing an inadequate number of cells expressing the SPEC gene to result in an antibiotic-resistant phenotype.

The spectinomycin test was only used to preselect those transformed lines in which there was most likely to be a significant level of *Ac* activity. It did not guarantee that any *Ac* copies were still present at sites close to the T-DNA. Because of the well-known tendency of this transposon to re-insert at linked sites it was likely, but not certain, that some transposed *Ac*s (tr*Ac*s) would be found to cluster around the T-DNA. It was envisaged that it would be necessary to obtain evidence of the presence of these linked tr*Ac*s prior to commencing a targeted tagging experiment with any of these transformed lines. However, it was only necessary to do this for those transformants in which the T-DNA had been shown to be linked to the target gene. The next step in the strategy was therefore to identify transformants where the T-DNA had been inserted into the required regions of the tomato genome, i.e. the short arm of chromosome 1 and the long arm of chromosome 7.

Screening for T-DNA linkage to marker genes

An initial sample of 93 diploid primary transformants were backcrossed to the homozygous triple mutant (*au au, ls ls, pl pl*). Since each primary transformant was in a genetic background heterozygous at the *au*, *ls*, and *pl* gene loci, each backcross population was composed of plants segregating for all three mutant phenotypes. Small backcross populations of around 200 plants of a sample of 16 of these putative diploid transformants were grown and sprayed with kanamycin to confirm the presence of the T-DNA construct and to allow linkage of the T-DNA with any one of the marker genes to be assessed. The more tightly linked

Fig. 2 T-DNA::*Ac* construct SLJ591 used to transform heterozygous triple mutant cotyledons. The approximate positions and orientations of the primers are indicated by *arrows*; primer names are written *above* them. Primer sequences (5′–3′) are given *below*. SLJ591 was kindly donated by Dr. J. Jones, JII, Norwich). B34 ACGGTCGGTACGGGATTTTCCCAT, B35 TATCGTATAACCGATTTTGTTAGTTTTATC, B39 TTTCGTTTCCGTCCCGCAAGTTAAATA, B42′ CCATCTAGTTGAGACATCATATGAGATC, C10 AACATCCAACGTCGCTTTCAGGGATC, C11 CTCAGATACTTACGTCACGTCTTGC, C13 ATCTCCACTGACGTAAGGGATGACG, C14 AGTTGAGTCGATACTTCGGCGATCAC, C29 AAATTCAGGCCCGGTT GCCAT, C30 GCGCTTAGTACATGGTCGATAAG C31 GGGGCATCGCACCGGTGAGTAA, C32 TTGTGGGCCTGTGGTCTCAAGATGG, D14 AGGTAATGGGCTACACTGAATTGG, D43 CGCATAATCTCAGACCAATCTGAAG, D71 CCGTTACCGACCGTTTTCATCCCTA, D73 TTCCCATCCTACTTTCATCCCTG, E4 GGTAAACGGAAACGGAAACGGTAG, Ac1 CTGACCACTATGCTTCAAAAGGGG, Ac2 CCAATTCATTTGACTCTACTTGATC, Ac3 GGCATCCCTCAACATCAAAT AGG. To amplify DNA flanking left border T-DNA, primers D14 and C29 were used followed by a nested amplification using primers D43 and C11. For right border amplifications, primers C30 and C32 were used followed by a nested amplification using primers C10 and C31. Amplifications of DNA flanking transposed *Ac* elements used: for the 5′ end, primers B34 and B35 followed by a nested amplification using primers D73 and E4 and for the 3′ end, primers Ac1 and B39 followed by a nested amplification using primers Ac2 and D71



the T-DNA to a mutant marker, the more frequently the two phenotypes cosegregated either in coupling or repulsion phases. No linkage resulted in a random distribution of kanamycin resistance, with respect to the mutant phenotypes, throughout the backcross population.

Data from these backcrosses (some of which is shown in Table 1) indicated that in 1 transformant (T103) the T-DNA had been inserted relatively close to (17.1 cM) the mutant *au* allele on the short arm of chromosome 1 and in another (T160) was linked to *ls* (36.3 cM) on the long arm of chromosome 7. This relatively simple system allowed the identification of transformants in which the T-DNAs were located in the preselected regions of the tomato genome of interest. Confirmation of the linkage to *au* of the T103 T-DNA was obtained by backcrossing, to an *au* homozygote, a recombinant plant from the original backcross test population in which the T-DNA was on the same homologue as the wild-type allele at the *au* gene locus. This population gave a recombination distance of 15.4 cM between *au* and the T-DNA (Table 1). The position of the T-DNA could have been either distal or proximal to *au*. To define its position a three-point testcross was carried out between *au*, the kanamycin resistance gene of the T-DNA (Kan R) and the dominant mutant *Lapageria* (Table 1). These segregation data showed that the T-DNA was approximately mid-way between *Lpg* and *au* (see Fig. 1). The double recombinants (+ KanS *au* and *Lpg* KanR +) from this three-point test cross were useful in providing some evidence that plants derived from transformant line T103 retained copies of the transposon (tr*Ac*s) that were still linked to the T-DNA. Southern analysis of 4 plants each of the two double recombinant genotypes, using an *Ac*-specific probe (Fig. 5), showed that tr*Ac*s were preferentially found in the *Lpg*-containing double recombinants (all 4 plants) compared with the reciprocal *au* genotype (only 1 plant with tr*Ac*s). This was exactly the opposite of the situation previously detected in non-recombinant genotypes where copies of the transposon were preferentially encountered in association with the mutant allele *au* and not with *Lpg*. It appeared that many of the tr*Ac*s had moved from one homologue of chromosome 1 to the other in association with recombination of the region around the T-DNA. It was clearly important to have this information before contemplating using this material in a targeted transposon mutagenesis experiment.

Inverse PCR of T-DNA flanking regions

Forty-two diploid, kanamycin-resistant, transformed lines including those whose F_2 populations segregated 3:1 for resistance to spectinomycin (likely therefore, to contain only one T-DNA::*Ac* copy with active *Ac* elements) were subjected to molecular mapping procedures to identify the RFLP map locations of their T-DNAs. Most of these transformed lines had shown no linkage to any of the three marker genes in classical mapping studies; hence these T-DNAs were assumed to have inserted in positions distant from the marker genes or onto other chomosomes. Transformant lines T103 and T160 were also included in these analyses in attempts to confirm the locations of their T-DNAs by RFLP mapping.

In 71% of the transformants, an authentic IPCR fragment was generated from at least one border of the T-DNA. Authenticity of the amplified product was checked firstly by using a nested set of primers which, for authentic products, resulted in a predicted shift in electrophoretic mobility (about 140 bp for the left border and 100 bp for the right border) and secondly by sequencing the termini of IPCR products. As expected, such DNA sequencing showed the transfer of significant tracts of the T-DNA border to the transformant which for left border fragments was often relatively long and of variable length (Fig. 3). All mapped T-DNAs possessed unique genomic junction fragments.

RFLP mapping

Southern hybridisation analyses revealed that 27 of the T-DNAs for which inverse PCR fragments were generated had integrated into single-copy genomic DNA;

Table 1 Segregation data in backcross populations of transformant lines showing linkage to marker genes

Markers	Method[a]	Recombination (%)	Map distance (cM)[b]	χ^2 (1:1:1:1)	Number of progeny
T-DNA(T103)/*au*	CT	16.5	17.1	295.3*	654
T-DNA(T103)/*au*	RT	14.9	15.4	179.2*	357
T-DNA(T160)/*ls*	RT	31.0	36.3	51.4*	342
T-DNA(T103)/*Lpg*	R3	15.6	16.1	192.2*	405
T-DNA(T103)/*au*	C3	14.8	15.6	200.5*	405
Lpg/*au*	R3	30.4	35.3	84.5*	405

* Values are χ^2 summations significant at $P < 0.05$

[a] CT, RT: result of two-point testcrosses in coupling and replusion phases, respectively, C3, R3: results of three-point testcross with markers in coupling and repulsion phase, respectively

[b] Map distances (cM) calculated using Kosambi mapping function

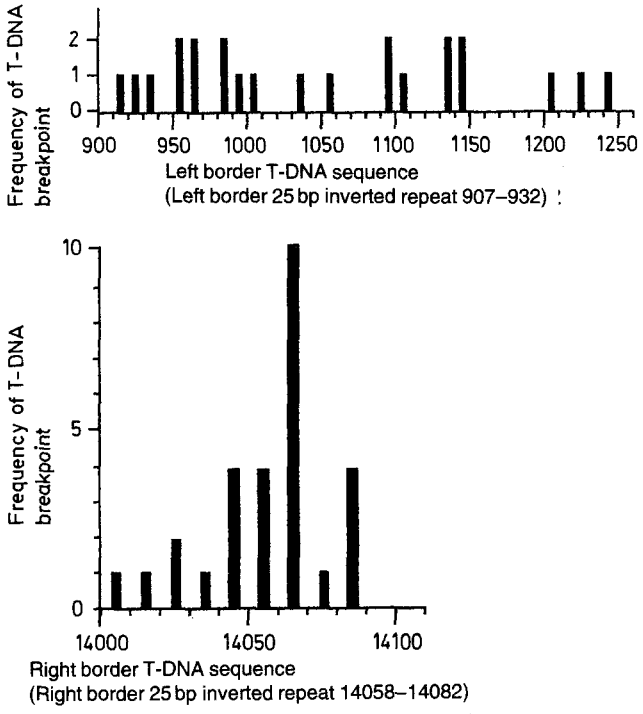


Fig. 3 Distribution of 'T-DNA breakpoints' throughout the left and right border sequences. Right border breakpoints were concentrated into a relatively short sequence of DNA (90 bp) when compared with the left border breakpoints (1240 bp)

IPCR fragments from 5 transformant lines hybridised to multicopy DNA sequences and 1 hybridised to a multigene family.

The RFLP map positions of the T-DNAs were determined using an F_2 population of *L. esculentum* × *L. pennellii*. Numerous cDNA and genomic clones have been placed on the RFLP map using this F_2 population (Tanksley et al. 1992) in conjuction with the computer programme Mapmaker (Lander et al. 1988).

Radio-labelled IPCR clones were hybridised to Southern filters of digested F_2 DNAs to give RFLP patterns. The data from these Southerns were entered into the Mapmaker programme, which compared the new dataset with data from previously mapped markers to determine the likely position of the new marker on the RFLP map.

Twenty-three T-DNA insertion sites were RFLP-mapped from 21 independent diploid transformants (Fig. 4). There seemed to be no obviously favoured regions of the genome for T-DNA integration. A similar random distribution of T-DNA integration sites was reported by Thomas et al. (1994). Transformant T254 regenerated too late to be included in the marker gene linkage programme. Molecular mapping of its T-DNA flanking regions indicated the presence of two T-DNA insertions; one RFLP mapped to chromosome 1 close to the marker TG24 that co-maps with *au* (Balint-Kurti et al. 1994); the other T-DNA was located on chromosome 6 (Fig. 4).

Molecular verification of classical mapping results

The selection scheme identified two transformants, each of which carried one T-DNA::*Ac* copy linked to mutant marker genes. The T-DNA of transformant T160 was linked to the marker gene *ls* on chromosome 7. An IPCR fragment was amplified from the left border of this T-DNA and was subsequently mapped onto the RFLP map of chromosome 7. The T-DNA of transformant T103 was linked to the marker gene *au* on chromosome 1. Unfortunately, an IPCR fragment from T103 could not be detected, possibly because the religated *Bst*YI- and *Bcl*I-digested DNA samples contained T-DNA-flanking genomic DNA that was too long to amplify by inverse PCR under the standard conditions. T103 was preselected on the basis that the T-DNA insertion site was linked to the marker gene *au* on chromosome 1 (Table 1) and therefore was of particular interest. In order to provide further evidence for the linkage of this T-DNA to *au* (and consequently *sit*) on the RFLP map, inverse PCR techniques were used to generate tomato genomic fragments that flanked *Ac* transposition/re-insertion events.

A number of the IPCR fragments generated from copies of *Ac* in T103 were used as probes in mapping experiments using the Tanksley *L. esculentum* × *L. pennellii* F_2 population. Three RFLP-mapped tr*Ac*s were on other chromosomes. One of these was on chromosome 3; the other two were linked on chromosome 5 (Fig. 4). Three further *Ac* copies were in a very tight cluster at a position 18 cM proximal to RFLP marker TG24 on chromosome 1 (Fig. 4). It was probable that this tight cluster of tr*Ac*s occurred close to the T-DNA insertion site and could be taken to represent the RFLP map location of the T-DNA insertion site. Since the marker gene *au* is known to map very close to TG24 (Balint-Kurti et al. 1994), these data agreed well with a weighted classical map distance of 16.2 cM separating the T-DNA of transformant T103 and *au* (Table 1). The pattern of 50% of tr*Ac*s to linked sites and 50% to unlinked sites has been reported elsewhere (e.g. Osborne et al. 1991). The RFLP mapping of the tr*Ac* cluster (*Ac* 1, 2 and 3 – Fig. 4) around the T-DNA of T103 provided independent support for the conclusion from Southern analysis of double recombinant genotypes (Fig.5). Both observations indicated that this line had retained copies of the transposon linked to the T-DNA and was therefore suitable for a targeted transposon mutagenesis programme.

Discussion

The work presented here, in which a marker gene system was used, has shown that the preselection of primary transformants that carry T-DNA::*Ac* cassettes and show linkage to specified target loci is a possible means to initiate a targeted transposon tagging strategy. Tomato has an extensively mapped genome (Mutschler et al.

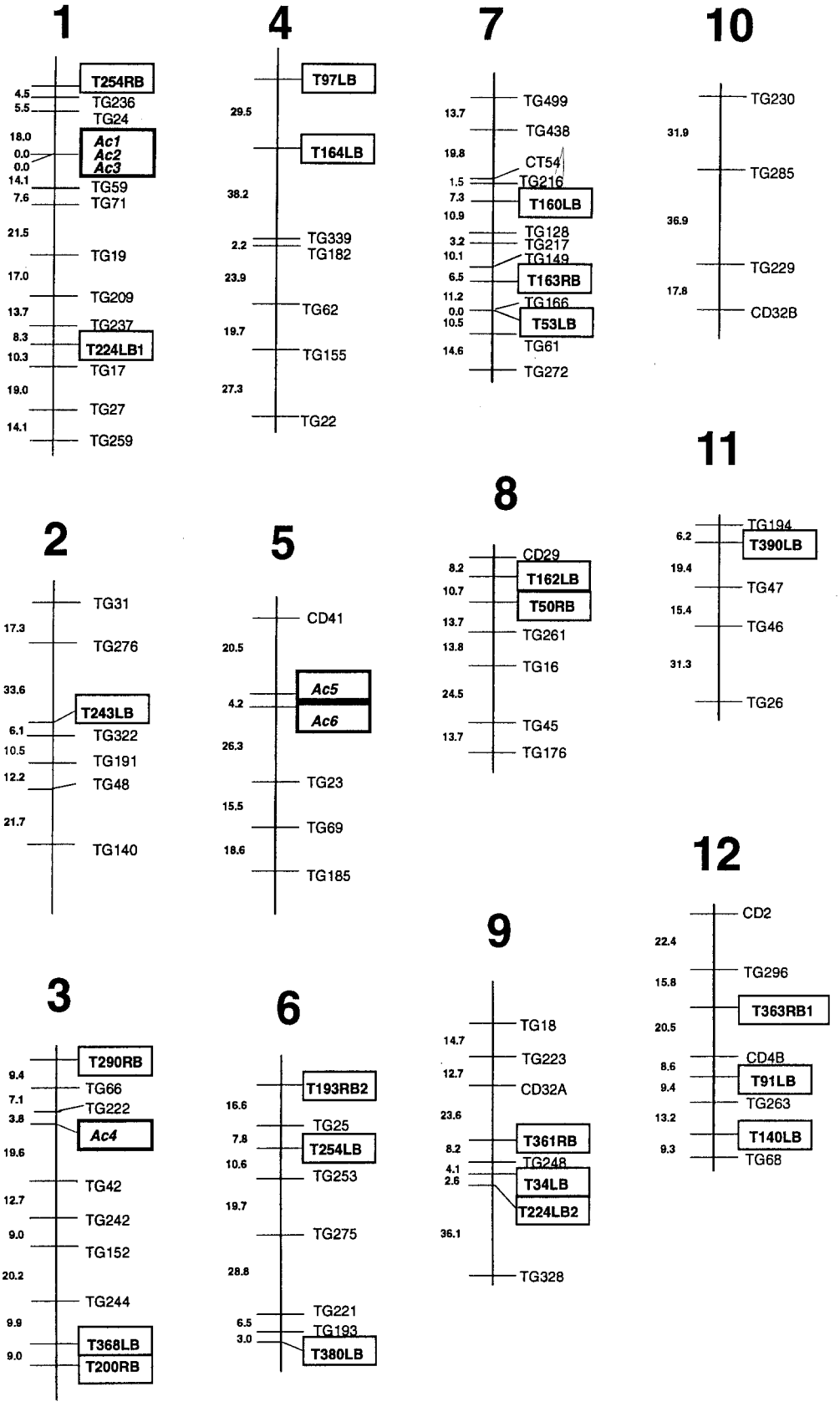


Fig. 4 RFLP maps of the 12 tomato chromosomes. Maps were generated using the programme MAPMAKER (Lander et al. 1987). Map distances between markers are shown in cM. The marker gene *au* co-maps with TG24 on chromosome 1. T-numbers in *boxes* refer to the RFLP positions of T-DNAs in primary transformant lines; *LB* (left border) and *RB* (right border) indicate which T-DNA flanking fragment was used to map the T-DNA. *Ac* numbers in *boxes* indicate the positions of secondary transposon re-integrations; *unboxed* markers are Tanksley RFLP probes (Tanksley et al. 1992). LOD scores for T-DNA and *Ac* map positions: T254RB 6.92, *Ac*1 3.37, *Ac*2 3.37, *Ac*3 3.37 T224LB1 4.02, T243LB 2.64*, T290RB 5.17, *Ac*4 7.13, T368LB 3.94, T200RB 7.69, T97LB 1.75, T164 0.99, *Ac*5 3.33, *Ac*6 5.43, T193RB2 3.87, T254LB 4.37, T380LB 11.29, T160LB 3.72, T163RB 5.13, T53LB 9.03, T162LB 4.25, T50RB 1.56*, T361RB 1.84*, T34LB 6.07, T224LB2 4.67, T390LB 8.23, T363RB1 3.45, T91LB 6.62, T140LB 5.93. * LOD scores for linkage of flanking RFLP markers (Tanksley et al. 1992) to each other were below 3.00; these low LOD scores may account for low LOD scores for *asterisked* markers *N.B* Integration of classical and RFLP maps has been carried out in detail for few tomato chromosomes (e.g. chromosome 6; Weide et al. 1993). This makes it difficult to compare Fig. 4 with Fig. 1. The fact that classical linkage tests indicated that T160 was more closely linked to *ls* than either T163 or T53 emphasises this point. This result suggests that the classical and RFLP maps of chromosome 7 have been incorrectly orientated. It is now known that *ls* maps close to the RFLP marker TG499 (Schumacher et al. 1995). Data giving a more complete alignment of the two maps of chromosome 7 will form the basis of a forthcoming paper

1987; Tanksley et al. 1992). For RFLP mapping strategies it is necessary to know the map position of many DNA markers. Not only is a well-characterised genome required but, to obtain meaningful map data, the same population of mapping plants should ideally be used for all DNA markers. Such information is not required if

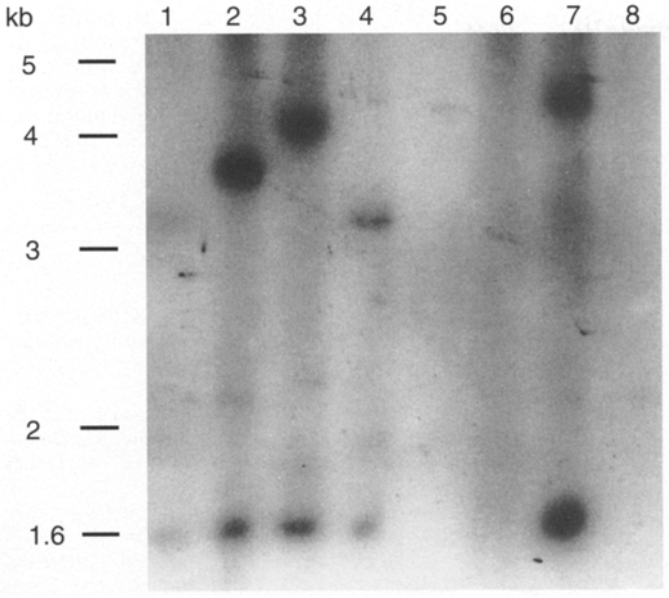


Fig. 5 Southern analysis of tr*Ac* copies in double recombinants from the *Lpg, au*, T-DNA three-point testcross population (see Table 1). *Lanes 1–4* individual double recombinants retaining the T-DNA (*Lpg*, T-DNA, +); *lanes 5–8* individual double recombinants lacking the T-DNA (+, +, *au*). DNA samples were digested with *Hin*dIII to yield a 1.6-kb *Ac* internal fragment and *Ac* 5′ unique junction fragments. The probe was generated by PCR from an *Ac*-containing plasmid and spanned part of the 5′ and central regions of the *Ac* element

classical linkage can be determined between the T-DNA and conventional mutant genes. For example, the classical map positions of the gravitropic tomato mutants (*lazy* 1 and *lazy* 2) are unknown; however, a targeted transposon tagging strategy could be developed by transforming T-DNA::*Ac* constructs into lines heterozygous for the *lazy* mutant alleles. T-DNAs linked to these *lazy* mutants could be selected by classical segregation analysis in backcross populations in a procedure parallel to that used in this paper. The use of an RFLP approach to identify T-DNA::*Ac* constructs linked to the *lazy* mutants would require knowledge of their classical (and RFLP) map positions that is not currently available (Mutschler et al. 1987).

A further advantage of the classical mapping/tagging strategy is that it does not rely on the accuracy of pre-existing mapping data. The *Cladosporium fulvum* resistance genes *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9* of tomato were believed to be located on chromosomes 6 (Langford 1937), 1 (Kerr and Bailey 1964), 4 and 10 (Kanwar et al. 1980). RFLP/IPCR mapping strategies would have selected T-DNAs mapping to these chromosomes. However, recent evidence has shown that some of these map positions are incorrect (Balint-Kurti et al. 1995). It is now known, for example, that *Cf-9* is not located on chromosome 10 but on chromosome 1 (Balint-Kurti et al. 1995). Hence, RFLP mapping strategies relying on the old classical map would have selected T-DNA::*Ac* lines that were unlinked to *Cf-9*. The direct use of linkage between the target locus and T-DNA in transgenic lines avoids these problems, allowing direct selection of useful transformant lines with a minimum of accurate background information.

The procedure of transforming tissue which is heterozygous for the target gene (or a cosegregating marker gene) has the added benefit that it selects all transformants that show linkage. This is not always the case with the IPCR and RFLP approach where reliance is placed on being able to generate an IPCR fragment suitable for radiolabelling in Southern analyses. The procedure also depends on the RFLP map being complete enough to allow mapping of the IPCR fragment. Exclusive use of molecular mapping in our system would have resulted in the failure to either detect or map the T-DNA of transformant line T103. Placing this T-DNA onto the RFLP map of tomato was only possible by an indirect method. Several *Ac* re-insertions were located on the RFLP map; clustering of some of these events gave an indication of the most likely position of the T-DNA.

Any readily transformable plant species in which a suitable mutant already exists could potentially be used in a targeted tagging preselection system similar to the one reported here. Transformants could be generated in a genetic background heterozygous for the target gene as long as the mutant allele was subsequently selected away before a tagging programme was initiated. In such systems transformants showing classical linkage of T-DNA to the target gene locus could be identified, isolated and subsequently used in a targeted transposon tagging screening programme. A species such as *Nicotiana plumbaginifolia*, which is easy to transform, has interesting mutants (e.g. Rousselin et al. 1992) and yet lacks an integrated classical and RFLP map, would be ideal for this approach.

The value of this method is clearly demonstrated in Table 1. No apparent preference was shown by introduced T-DNAs for any section of the genome. This result supports that reported by Thomas et al. (1994) and implies a broadly equal probability of integration throughout the transcribed region of the genome. The best estimate for the genome size of tomato can be obtained from the RFLP map, which is believed to span around 1400 cM (Tanksley et al. 1992). A T-DNA insertion 35 cM either side of a target gene (which is at least 35 cM from the end of the chromosome) would occur with an average probability of 70/1400, i.e. 1 in 20. If, as in the case here, there is more than one target gene, the probability of obtaining a transformant carrying a T-DNA less than 35 cM from one of the target genes is increased. By using an initial sample of 16 primary transformants to screen for linkage between the T-DNA and the marker genes, we were perhaps a little fortunate to obtain evidence of insertions 36.3 cM from *ls* and 16.2 cM from *au*. However, we could have easily continued this process with our remaining transformants if this had been necessary. The analysis of this number of transformants by molecular techniques is costly and time-consuming. However, the relatively rapid (and cheaper) screening using a marker gene in a heterozygous background for transformation allows easy

selection of transformants close to the target site. This approach potentially opens up the targeted transposon tagging strategy to many other species for which neither classical nor RFLP maps exist. Even in tomato, the incomplete integration of classical and RFLP maps presents difficulties in locating the T-DNAs that are closest to a target gene (see Fig. 4 legend).

Of the total number of diploid putative transformants available for molecular analyses (IPCR/RFLP), not all had been previously subjected to classical linkage analysis. One of these untested lines, T254, regenerated too late to be included in the marker gene linkage programme. Inverse PCR of DNA from this transformant line gave two fragments: one was located close to TG24 (which co-maps with the marker gene *au*; Balint-Kurti et al. 1994; the other mapped to chromosome 6. The screening procedure involving the three marker genes could not easily have been employed with transformants carrying two T-DNA::*Ac* cassettes, such as T254.

The results presented here demonstrate advantages and limitations to both systems. Direct screening for linkage to mutants has major benefits. It does not rely on the existence or accuracy of classical or RFLP maps and transformant lines that have a T-DNA linked to a target or co-segragating marker gene can be selected. On the other hand, as a result of work presented here and elsewhere (e.g. Thomas et al. 1994; Rommens et al. 1993) there are mapped *Ac* and *Ds* primary insertions scattered over the tomato genome. The RFLP map of tomato is now extensive and the map positions of the markers precise (Tanksley et al. 1992). This information can allow the selection of transposon-carrying lines that are linked to target genes whose location is accurately known. Using this method, Jones et al. (1994) have successfully transposon-tagged the *C. fulvum* resistance gene, *Cf-9*, in tomato. By first classically mapping *Cf-9* to the short arm of chromosome 1 and then integrating this with the RFLP map (Balint-Kurti et al. 1995), it was possible to select a *Ds*-carrying line that mapped close to *Cf-9*. Transformant line FT33 (Rommens et al. 1993) was chosen because its integrated map position was very close (3 cM) to that for *Cf-9* (Balint-Kurti et al. 1995). In most cases such a conveniently close *Ds* element would not currently be available. In these circumstances, it may be necessary to induce further transposition and re-insertion of *Ds* elements from more distantly located T-DNAs before attempting to tag a gene of interest. It should be noted that the *Ds* element referred to in transformant line FT33 is on the short arm of chromosome 1, i.e. the same region as the *au* and *sit* loci. This *Ds* element is currently being used along with the tr*Ac* element-containing line (T103) described in this paper in transposon mutagenesis programmes involving these target genes. The relative merits of dual element (*Ds*) versus single element (*Ac*) transposon-tagging strategies in tomato will be considered in the light of this experience.

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