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Published in:
MGG Molecular %26 General Genetics

DOI:
10.1007/BF02172517

Publication date:
1996

Mol Gen Genet (1996) 251:267–280

ORIGINAL PAPER

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Identification and isolation of the *FEEBLY* gene from tomato by transposon tagging

Received: 18 September 1995 / Accepted: 17 November 1995

Abstract The *Ac/Ds* transposon system from maize was used for insertional mutagenesis in tomato. Marker genes were employed for the selection of plants carrying a total of 471 unique *Ds* elements. Three mutants were obtained with *Ds* insertions closely linked to recessive mutations: *feebly* (*fb*), *yellow jim* (*yj*) and *dopey* (*dp*). The *fb* seedlings produced high anthocyanin levels, developed into small fragile plants, and were insensitive to the herbicide phosphinothricin. The *yj* plants had yellow leaves as a result of reduced levels of chlorophyll. The *dp* mutants completely or partially lacked inflorescences. The *fb* and *yj* loci were genetically linked to the *Ds* donor site on chromosome 3. Reactivation of the *Ds* element in the *fb* mutants by crosses with an *Ac*-containing line resulted in restoration of the wild-type phenotypes. Plant DNA fragments flanking both sides of the *Ds* element in the *fb* mutant were isolated by the inverse polymerase chain reaction. Molecular analysis showed that phenotypic reversions of *fb* were correlated with excisions of *Ds*. DNA sequence analysis of *Fb* reversion alleles showed the characteristic *Ds* footprints. Northern and cDNA sequence analysis indicated that transcription of the *FEEBLY* (*FB*) gene was impeded by the insertion of *Ds* in an intron. Comparison of the predicted amino acid sequence of the FB protein with other database sequences indicated that *FB* is a novel gene.

Key words Transposon tagging · *Activator/Dissociation* · *Lycopersicon esculentum* · Mutants

Communicated by H. Saedler

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Introduction

The classical genetics of tomato (*Lycopersicon esculentum*) is well developed and has resulted in a large collection (>1200) of monogenic mutants (Stevens and Rick 1986). Functional analysis of the genetic defects would be greatly facilitated if the corresponding genes were cloned. Insertional mutagenesis using T-DNAs or transposons permits identification of mutant phenotypes and isolation of the corresponding genes (Walbot 1992). An advantage of transposons is that tissue culture-induced mutations can be avoided by screening for mutant phenotypes after several generations. Furthermore, gene isolation by transposon tagging requires no genetic complementation analysis: the authenticity of the insertion mutant can be verified by reversion analysis. Part of the gene flanking the transposon can be cloned by the inverse polymerase chain reaction (IPCR) and, subsequently, be used for the isolation of the corresponding cDNA (Van der Biezen et al. 1994b).

Endogenous transposons have successfully been used for mutagenesis and gene isolation in maize (*Zea mays*), snapdragon (*Antirrhinum majus*) and *Petunia hybrida* (Fedoroff et al. 1984; Martin et al. 1985; Souer et al. 1995). In plant species lacking well-characterized transposons, T-DNAs and heterologous transposons were used for the development of insertional mutagenesis strategies (Feldmann 1991; Haring et al. 1991). Using transposons from maize for transposon tagging in Arabidopsis and Petunia, unstable mutant phenotypes were identified first and, secondly, parts of the corresponding genes were isolated (Aarts et al. 1993; Bancroft et al. 1993; Chuck et al. 1993; Long et al. 1993). The effectiveness of these nontargeted transposon tagging experiments depended on the efficiency of selection for plants that had undergone transposition events and on the criteria for mutant designation. Maize transposons were also efficiently applied for the isolation of preselected target genes that were only

known by their phenotype in tomato (Jones et al. 1994), tobacco (*Nicotiana tabacum*) (Whitham et al. 1994), Arabidopsis (James et al. 1995), and flax (*Linum usitatissimum*) (Lawrence et al. 1995). The efficacy of these targeted transposon tagging procedures depended on the activity of the transposons and the genetic distances of the transposons from the target genes.

To assess the mutagenic potential of the *Activator* (*Ac*)/*Dissociation* (*Ds*) system in heterologous hosts, we recently estimated the relative contribution of the parameters involved and, subsequently, predicted the frequency of transposon-induced mutants (Van der Biezen et al. 1994b). In this study, we applied the *Ac*/*Ds* transposon system as an insertional mutagen in tomato to determine experimentally the frequency of transposon-induced mutants. Use was made of a stabilized *Ac* element (*sAc*) that functioned as a transposase donor, and reporter genes to monitor transposition of the nonautonomous *Ds* element. The procedure for recovery of *Ds*-induced mutants included four steps: (1) by simultaneous selection for excision and reinsertion, plants were identified that had inherited transposed *Ds* elements; (2) screening self progenies for deviant phenotypes revealed three families that segregated for single recessive mutations: *feebly* (*fb*), *yellow jim* (*yj*) and *dopey* (*dp*); (3) linkage analysis showed that the three mutant alleles were associated with distinct *Ds* insertions; (4) genetic analysis of *fb* mutants showed that excisions of the *Ds* elements were accompanied by phenotypic reversions. Molecular analysis showed that the *Ds* element was inserted in an intron and impeded transcription of the *FB* gene.

Materials and methods

Plant material, genetic crosses and linkage analysis

The tomato line ET570 harbored a single-copy *Ds*-containing T-DNA insertion that was mapped at the end of the long arm of chromosome 3 (Knapp et al. 1994; Van der Biezen et al. 1994a). This *Ds* element was marked with the hygromycin resistance gene (*HPT*) and the phosphinothricin (PPT) resistance gene (*BAR*). The SLJ10512 transformant carried a single-copy T-DNA insertion with an *sAc* and a β-glucuronidase *uidA* (*GUS*) reporter gene, and was localized on chromosome 4 (Thomas et al. 1994). Both constructs had been transferred to tomato cv Moneymaker and are schematically shown in Fig. 1. The F_2 population used for restriction fragment length polymorphism (RFLP) mapping was derived from the *L. esculentum* Tester-3 and the wild tomato *Lycopersicon pennellii* LA716 (Van der Biezen et al. 1995). All crosses were performed in the greenhouse using standard emasculation and pollination techniques. The F_1s were made by reciprocal crosses between the *sAc* and *Ds* parents. For reversion analysis, the mutants were backcrossed with the *sAc* line as the staminate parent. Plants were grown in pots under standard Dutch greenhouse conditions (20° C, 60% humidity, 3–6 klx). Recombination values and map positions were calculated using the computer package JoinMap (version 1.3) with a critical LOD score of 3.0 for linkage and 0.05 for mapping using Haldane's mapping function (Stam 1993).

Selection of seedlings carrying germinally transmitted *Ds* transpositions

The *Ds* excision (*BAR*) and insertion (*HPT*) markers were used for simultaneous selection of F_2 seedlings containing excised and reinserted *Ds* elements on medium with PPT and hygromycin (Hyg). However, PPT-resistant seedlings were not necessarily the consequence of germinal transpositions because the *BAR* gene acts in a non-cell-autonomous manner and, hence, PPT resistance could also be the result of somatic *Ds* excisions (Jones et al. 1993). To select indisputably seedlings harboring a germinally excised and reinserted *Ds* element, Hyg/PPT-resistant seedlings that did not contain the *sAc* element were selected by analysis of the expression of the *GUS* reporter gene that was linked on the same T-DNA (Scofield et al. 1992). This approach had the concomitant advantage that stably transposed *Ds* insertions (*trDs*) were selected, which simplified the molecular genetic analysis of mutants. For the calculation of the frequencies of *Ds* transpositions that were transmitted through the gametes of doubly hemizygous *Ac*/*Ds* F_1 plants, recall that only 1/4 of the F_2 seedlings that carried such events could be recognized unambiguously (1/4 GUS^-, 3/4 GUS^+) and, therefore, the germinal frequencies were based on 1/4 of the total seedlings. Estimation of the germinal transposition frequencies by selection against *sAc* required that the ET570 and SLJ10512 T-DNA insertions were genetically unlinked.

For aseptic seedling assays, F_2 seeds were dipped in 70% ethanol for 30 s, soaked in 1% NaClO (w/v) for 20 min and rinsed in sterile water. Following this sterilization procedure, 20–40 seedlings were placed in containers containing 50 ml Murashige-Skoog (MS) medium, pH 5.7 with 1.5% sucrose, 0.8% agar (Difco), 50 mg/l PPT (Finale SL14; Hoechst) and 20 mg/l hygromycin (Duchefa), and placed in a climate cabin under standard conditions (16 h, 3 klx, 22° C). Three weeks after germination, 5 ml 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) in 50 mM $NaHPO_4$, pH 7.0 was poured on the medium and after 16 h in the climate cabin, the roots of Hyg/PPT-resistant seedlings containing the *GUS* gene showed blue precipitates. Seedling classes were counted and the Hyg/PPT-resistant seedlings without GUS activity were selected and transferred to the greenhouse. The absence of the *sAc* T-DNA (SLJ10512) was verified in leaf tissue extracts from greenhouse-grown plants by fluorometric GUS analyses with 1 mM 4-methylumbelliferyl-β-D-glucuronide (MUG) (Jefferson et al. 1987) and by the polymerase chain reaction (PCR) with the *ac6*/*ac7* primers (Fig. 1).

Pigment determinations

To detect specific anthocyanins, fresh leaf tissues were incubated in 2 M HCl for 16 h and after hydrolysis (20 min at 100° C), the anthocyanins were extracted in a small volume of isoamylalcohol and applied on cellulose thin layer chromatography (TLC) plates using acetic acid : hydrochloric acid : water (3:3:10) as the developing solvent. Corolla tissues of *P. hybrida* lines were used as reference for the migration of specific anthocyanins (R. Koes, personal communication): *R*27 (cyanidin), *M*1 (peonidin), *V*41 (delphinidin), *V*48 (delphinidin), *V*26 (petunidin) and *VR* (malvidin).

For spectrophotometric analysis of the anthocyanin and chlorophyll contents, 100 mg leaf material was ground in liquid nitrogen and dissolved in 1 ml 1% (v/v) HCl in methanol or 1 ml 80% (v/v) acetone in water, respectively. Concentrations of serial dilutions were determined by absorbance measurements (LKB Ultrospec Plus) at the appropriate wavelengths: 652 nm (chlorophyll *a*/*b*) and 530 nm (anthocyanin). For the anthocyanin levels the formula $A_{530} - 0.25 \times A_{657}$ was used to correct for the chlorophylls and their degradation products (Rabino and Mancinelli 1986). Two extracts were made per plant and absorbance measurements were done in duplicate.

DNA isolation and hybridization

Four grams of leaf tissue was ground in liquid nitrogen, and after addition of 25 ml cold extraction buffer (0.35 M sorbitol, 0.1 M TRIS-HCl, 5 mM EDTA, pH 7.5, 20 mM $Na_2S_2O_3$), the mixture was centrifuged (10000 *g*) for 1 h at 4° C. Subsequently, 1.25 ml extraction buffer, 1.75 ml nucleus lysis buffer (2% w/v hexadecyltrimethylammonium bromide (CTAB), 0.2 M TRIS-HCl, 2 M NaCl, 50 mM EDTA), and 0.6 ml 5% w/v Sarkosyl were added, and following brief mixing the solution was incubated at 65° C for 1 h. The mixture was extracted with 7.5 ml chloroform : isoamylalcohol (24:1), the DNA was precipitated from the aqueous phase with an equal volume of isopropanol, and dissolved in 500 µl TE (10 mM TRIS-HCl, 1 mM EDTA, pH 8.0) with 5 µg RNase A at 60° C. After phenol : chloroform (1:1) extraction the DNA was precipitated with ethanol and dissolved in 1 ml TE.

Ten micrograms of total DNA was digested with restriction endonucleases (Boehringer) and the resulting fragments were size-fractionated by electrophoresis through 0.7% w/v agarose gels. After depurination, denaturation and neutralization, the DNA was transferred to Hybond-N^+ membranes by vacuum blotting (LKB) for 1 h with 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate), and fixed to the membrane by 0.4 M NaOH according to the supplier (Amersham). Hybridizations were carried out in 1% w/v bovine serum albumin (BSA), 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, 7% w/v SDS at 60° C for 16 h in a hybridization oven (Hybaid), using [α-^{32}P]dCTP (Amersham) radiolabeled probes prepared by random priming of gel-purified DNA or PCR products. Filters were washed with 2 × SSC, 0.1% SDS or 0.1 × SSC, 0.1% SDS at 60°–65° C and exposed to preflashed Kodak X-Omat AR films at −70° C with intensifying screens. Filters were reused after removal of the previous probe by boiling for 2 min in 0.5% SDS.

RNA isolation and hybridization

One gram leaf tissue was ground in liquid nitrogen, transferred to 2.2 ml tubes containing 750 µl phenol : chloroform (1:1) and 750 µl extraction buffer (0.1 M TRIS-HCl, pH 8.5, 0.1 M NaCl, 20 mM EDTA, 1% w/v Sarkosyl) and mixed. After a second phenol : chloroform (1:1) extraction the nucleic acids in the supernatants were precipitated with 750 µl isopropanol, and dissolved in 0.5 ml sterile water. Subsequently, 0.5 ml 4 M LiCl was added and the mixture was left on ice for 3 h. The RNA was pelleted by centrifugation, dissolved in 400 µl sterile water, ethanol precipitated and dissolved in 100 µl sterile water. Poly(A)$^+$ RNA was isolated from total RNA using the Oligotex-dT kit (Qiagen). Twenty micrograms total RNA or 1 µg poly(A)$^+$ RNA and RNA size markers (Promega) were separated through 16% v/v formaldehyde, 1.5% w/v agarose gels in 20 mM 3-[N-morpholino]-propane-sulfonic acid, pH 8.0 (MOPS), 5 mM sodium acetate and 0.5 mM EDTA. Subsequently, the RNA was transferred to Hybond-N^+ membranes by capillary blotting and crosslinked with 40 mM NaOH (Sambrook et al. 1989). Hybridizations and autoradiography were carried out as described for DNA.

Polymerase chain reaction

Plant DNA for PCR analysis was extracted from young leaf material obtained by punching a leaf disk with the cap of a 1.5 ml tube. The disks were squashed in 20 µl 0.5 M NaOH, the debris was spun down, 10 µl of the extract was added to 200 µl 100 mM TRIS-HCl, pH 8.0 and the solutions were mixed. PCRs were performed in 50 µl volumes with 10 µl plant extract or with 0.1 µg genomic DNA, 0.1 U *Taq* polymerase (Boehringer) with the supplied buffer, 0.2 mM dNTPs and 1.0 µM primers. The mixtures were subjected to 2 min at 94° C, followed by 35 cycles of 30 s at 94° C, 60 s at 60° C and 90 s at 72° C, which were concluded by 10 min at 72° C in a time-regulated thermoblock (Perkin Elmer Cetus). The primer sequences were: *35s*, 5′-GCCCAGCTATCTGTCACTTCA-3′, *ac6*, 5′-CGCTTGTTCCATGATGACC-3′ (1633–1651 of *Ac*; Kunze et al. 1987); *ac7*, 5′-GCTGAAGCCTCTTCTAGTCGG-3′ (1231–1251); *act5b*, 5′-GCAA-GCTTCTCGAGGGGAGAGAGGCAGAGCAGCG-3′ (313–332); *bar*, 5′-TATCCGAGCGCCTCGTG-CAT-3′; *fe3*, 5′-GGGGATCCGGGAGTTCCGTGATCTCTAGGC-3′ and *fe5*, 5′-GGGAATTCGACA-CGGGTGTTGAGCAG-3′ (Isogen Bioscience, Amsterdam).

Isolation of *Ds*-flanking plant DNA by the IPCR

DNA of the *fb* mutant was digested to completion by *Apo*I, *Hin*fI and *Sph*I to obtain sequences flanking the 3′ *Ds* terminus, and by *Eae*I, *Hin*fI and *Eco*RII for the isolation of plant DNA flanking the 5′ *Ds* terminus. After phenol : chloroform (1:1) extraction and ethanol precipitation, 0.5–2 µg/ml DNA was circularized by 10 Weiss U/ml T4 ligase (Boehringer) in ligation buffer (50 mM TRIS-HCl, pH 7.4, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 50 µg/ml BSA and 1 mM ATP) for 16 h at 16° C. Subsequently, the DNA was ethanol precipitated, resuspended in sterile water and subjected to two series of PCR amplifications (Knapp et al. 1994). For the first series, ~100 ng template DNA was amplified with the primer combination for the 3′ *Ds* end: LDs7 (4219–4238 of *Ac*; Kunze et al. 1987) and LDs8 (4373–4392), and for the 5′ *Ds* end: LDs1 (158–177) and LDs2 (195–214); in the second amplification, 50 nl of the PCR mixture was further amplified with the nested primers for the 3′ *Ds* end: LDs9 (4126–4145) and LDs10 (4532–4551), and for the 5′ *Ds* end: LDs3 (13–32) and LDs11 (225–244). The authenticity of the PCR products was verified by using the fragments as hybridization probes on filters containing *Eco*RI- and *Hin*dIII-digested DNA of plants that were homozygous or hemizygous for the *Ds* insertion, and plants that did not contain the *Ds* element. The IPCR products with most plant DNA sequences flanking the 3′ and 5′ *Ds* termini were designated FE3 and FE5, respectively (Fig. 8).

cDNA analysis and DNA sequencing

The two IPCR products (FE3 and FE5) that flanked the *Ds* element in the *feebly* (*fb*) mutant were used as radiolabeled probes to screen an unamplified cDNA library (5 × 10^4 clones) made from leaf RNA of tomato cv Moneymaker with the UNI-ZAP-XR Gigapack 2 cloning kit (Stratagene). The IPCR products and cDNA sequences were cloned in the pGEM3Z vector using *Escherichia coli* strain DH5α. DNA sequences were determined using a Taq Dye Cycle sequencing kit (Applied Biosystems), universal M13 dye primers and an automatic DNA sequenator (Applied Biosystems Model 373A). Recombinant DNA work was performed using standard procedures (Sambrook et al. 1989). DNA sequences were analyzed using the BlastX and FASTA network services. The GenBank Accession Number of the cDNA sequence is U35643, and that of the IPCR products is U35644.

Results

Plants with unique, stable, germinally transmitted, transposed *Ds* elements

For the generation of plants carrying *Ds* transpositions, the parental SLJ10512 and ET570 transgenic lines, harboring single-copy *Ac*- and *Ds*-containing T-DNA

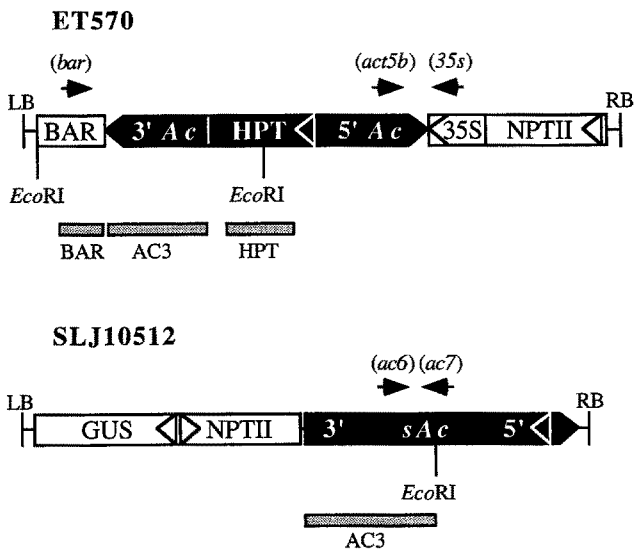


Fig. 1 T-DNA constructs with the nonautonomous *Ds* element (ET570) and the stabilized *Ac* (*sAc*) element (SLJ10512). The *Ds* element contained the hygromycin (Hyg) resistance gene *HPT* (hygromycin phosphotransferase), and had been inserted between the Cauliflower Mosaic Virus 35S promoter and the phosphinothricin (PPT) resistance gene *BAR* (phosphinothricin acetyltransferase). The *BAR* and *HPT* genes permitted simultaneous selection of plants with excised (PPT-resistant) and reinserted (Hyg-resistant) *Ds* elements. The *sAc* element was linked to a β-glucuronidase *uidA* (*GUS*) reporter gene. Both constructs carried left (*LB*) and right (*RB*) border sequences for transfer by *Agrobacterium* transformation, and the kanamycin resistance gene *NPTII* (neomycin phosphotransferase) for the selection of transformants. The *triangles* indicate the direction of transcription. The positions of the *Eco*RI restriction enzyme recognition sites and the hybridization probes for Southern analyses (*boxes*) are indicated below the constructs. The annealing sites of the oligonucleotides used as primers in polymerase chain reactions (PCRs) are shown as *arrows* (5′ → 3′) above the constructs

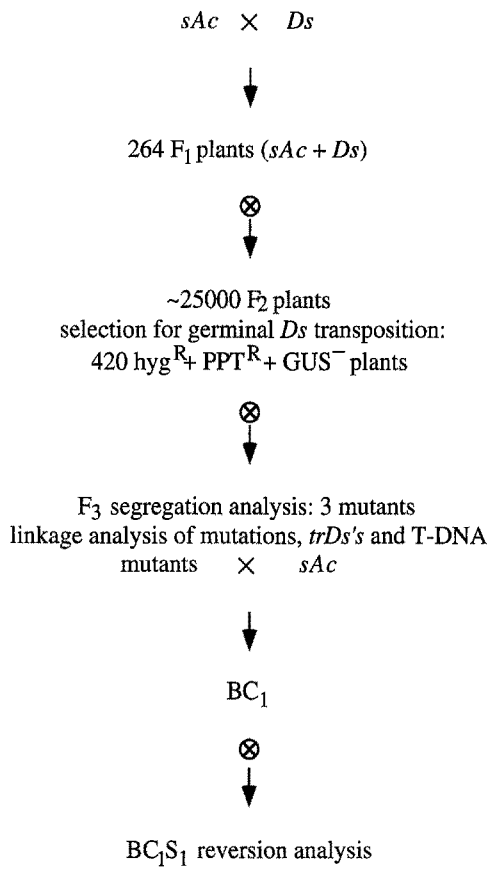


Fig. 2 Transposon tagging strategy for the generation and analysis of *Ds*-induced phenotypic mutants in tomato. The two parental lines were homozygous for single-copy T-DNA insertions with an *sAc* and *Ds* element, respectively, and were crossed to produce 264 doubly hemizygous *sAc*/*Ds* F_1 plants. *Ds* transpositions that occurred in the germ line were transmitted by self-pollination to F_2 progenies. Simultaneous selection for Hyg/PPT-resistant and GUS^- seedlings yielded 420 plants carrying stable, germinally transmitted and transposed *Ds* element insertions. To characterize the *Ds* transposition behavior in a subset of the total population, 90 F_2 plants from 24 F_1 plants were molecularly analyzed. To reveal whether the *Ds* elements were inserted in genes whose inactivation results in deviant visible phenotypes, 420 F_3 progenies were screened for segregation of mutants. In addition, the F_3 populations permitted analysis of the genetic linkage between the T-DNA, the *Ds* elements and the mutations. To analyze whether excisions of the closely linked *Ds* elements in the mutants led to restoration of the wild-type phenotypes, backcrosses (BC_1) were made to the *sAc* line, and the BC_1S_1 progenies were screened for somatic and germinal reversions

constructs, respectively, were used (Fig. 1). The *Ds* element was marked by the *HPT* gene and was cloned between the promoter sequences (*35S*) and the PPT resistance gene (*BAR*). This *Ds* element was somatically transactivated by the *sAc* element in the F_1 (Fig. 2). Transposed *Ds* elements were germinally transmitted through the F_1 gametes to F_2 progenies by self-pollination. Subsequently, simultaneous selection for Hyg/PPT resistance was applied to identify F_2 seedlings that carried excised (*BAR*) and reinserted (*HPT*) *Ds* elements. To distinguish germinal transposition from somatic *Ds* transposition events, seedlings were analyzed for the presence of the transposase donor (*sAc*) T-DNA by determination of GUS activity.

For detailed analysis of the germinal *Ds* transpositions in a subset of the total plant population, the F_2 progenies from 24 F_1 plants were separately harvested and subjected to Hyg/PPT and GUS assays (Table 1). Ninety Hyg/PPT-resistant and GUS^- F_2 seedlings were obtained and were molecularly analyzed (data not shown). The *Ds* excisions from the T-DNA were confirmed by PCR analysis with the *35s* and *bar* primers (Fig. 1): all 90 plants showed the empty donor site (EDS) fragments (0.8 kb). In addition, PCR with the *35s*/*act5b* primer combination showed in 37 cases (41%) the 0.6 kb full donor site (FDS) fragment. The presence and absence of *Ds* within the T-DNA was confirmed by Southern analysis of *Eco*RI-digested DNA and hybridized with the BAR probe (6.7 kb EDS fragment and 3.4 kb FDS fragment). To verify the reinsertion of the germinally transmitted *Ds* elements (*trDs*) and to analyze the incidence of independent *Ds* transpositions, *Eco*RI-digested DNA of all 90 plants was hybridized with the *Ds*-specific probe AC3 (Fig. 4a, middle panel). Twelve plants (13%) did not contain

Table 1 Selection and analysis of F_2 plants with germinally transmitted transposed *Ds* elements and determination of independent transposition frequencies

Family[a]	Total	$Hyg^R + PPT^R + GUS^-$[b]		Transposed *Ds*[d]		Independent transposition frequency %[e]
		Number	[%]	Unique	Total	
SE-1	313	3	1.0	5	5	6.4
SE-2	278	1[c]	0.4	0	0	0.0
SE-3	239	2	0.8	2	2	3.3
SE-4	281	3	1.1	4	5	5.7
SE-5	274	5	1.8	6	6	8.8
SE-6	150	2[c]	1.3	1	1	2.7
SE-7	173	6	3.5	8	8	18.5
SE-8	77	0	0.0	–	–	0.0
SE-9	401	1	0.2	1	1	1.0
SE-10	281	1	0.4	5	5	7.1
SE-11	265	11[c]	4.2	9	11	13.6
SE-12	241	8[c]	3.3	7	9	11.6
SE-13	324	14	4.3	23	27	28.4
SE-14	130	0	0.0	–	–	0.0
SE-15	56	1	1.8	1	1	7.1
SE-16	62	0	0.0	–	–	0.0
SE-17	66	0	0.0	–	–	0.0
SE-18	88	3	3.4	3	3	13.6
SE-19	133	8[c]	6.0	3	3	9.0
SE-20	203	6	3.0	6	6	11.8
SE-21	130	11	8.5	13	26	40.0
SE-22	124	1	0.8	2	2	6.5
SE-23	137	0	0.0	–	–	0.0
SE-24	25	3	12.0	4	4	64.0
Overall	4451	90	2.0	101	123	9.1

[a] F_2 plants derived from SLJ10512 × ET570 (SE) and subsequent self pollination of the F_1s
[b] For the selection of plants with stable, germinal *Ds* transpositions, seedlings were tested for resistance to phosphinotricin (PPT^R), resistance to hygromycin (hyg^R), and GUS activity
[c] Families that contained plants that did not carry transposed *Ds* elements. These plants were homozygous for the T-DNA: one allele conferred hyg^R (full donor site), the other PPT^R (empty donor site)
[d] The presence and number of different *Ds*s per plant was determined by Southern hybridization of *Eco*RI-digested DNA with a *Ds*-specific probe (AC3); different hybridizing restriction fragments per F_2 family indicated unique transposition events
[e] The frequency with which independent transpositions were germinally transmitted was based on 1/4 of the total population because only this proportion of seedlings with germinal *Ds* transpositions could be selected (GUS^-)

trDs elements and had been selected because Hyg/PPT resistances were conferred by FDS and EDS T-DNAs on the respective chromosome homologs. Unique *Ds* transposition events were distinguished for the F_2 families by comparing the sizes of the *Eco*RI restriction fragments that hybridized to the AC3 probe (>2.5 kb). Restriction fragments of different sizes indicated different, and thus, independently transposed *Ds* elements (Table 1). The 78 plants carried a total of 123 *trDs* elements (1–5 per plant), of which 101 were unique (82%). Accordingly, it was estimated that the average frequency of germinally transmitted, independent *Ds* transpositions was 9.1%. In addition to the primary *Ds* transpositions from the T-DNA, secondary transpositions could also have contributed to the high proportion of unique *Ds* insertion events. Conceivably, the frequency of independent *Ds* transpositions may be an overestimate.

To obtain a higher number of plants with *trDs* elements, 240 additional F_1 plants were self-pollinated and all ~21 000 F_2 seeds were pooled and analyzed for Hyg/PPT resistance and GUS activity. The large number of F_1 plants with the small average F_2 population size (~90 per family) increased the probability of selecting unique *trDs* elements. From this large F_2 population, 330 Hyg/PPT-resistant and GUS^- seedlings were selected (1.6%). Based on the molecular analysis of the 90 Hyg/PPT-resistant and GUS^- plants obtained in the pilot experiment (13% without *trDs*, 1.3 unique *trDs* per plant), it was estimated that these 330 plants carried in total 370 unique *trDs* elements.

Identification of mutant phenotypes

In total 420 F_2 plants were selected that carried an estimated number of 471 unique, stable and germinally transmitted *trDs* elements. All 420 F_2 plants were self-pollinated and the F_3 progenies ($n = 28$) were screened for segregation of severe visible mutant phenotypes. Three families were identified that segregated in a Mendelian fashion for single recessive mutations and these were designated *feebly* (*fb*), *yellow jim* (*yj*) and *dopey* (*dp*) (Fig. 3).

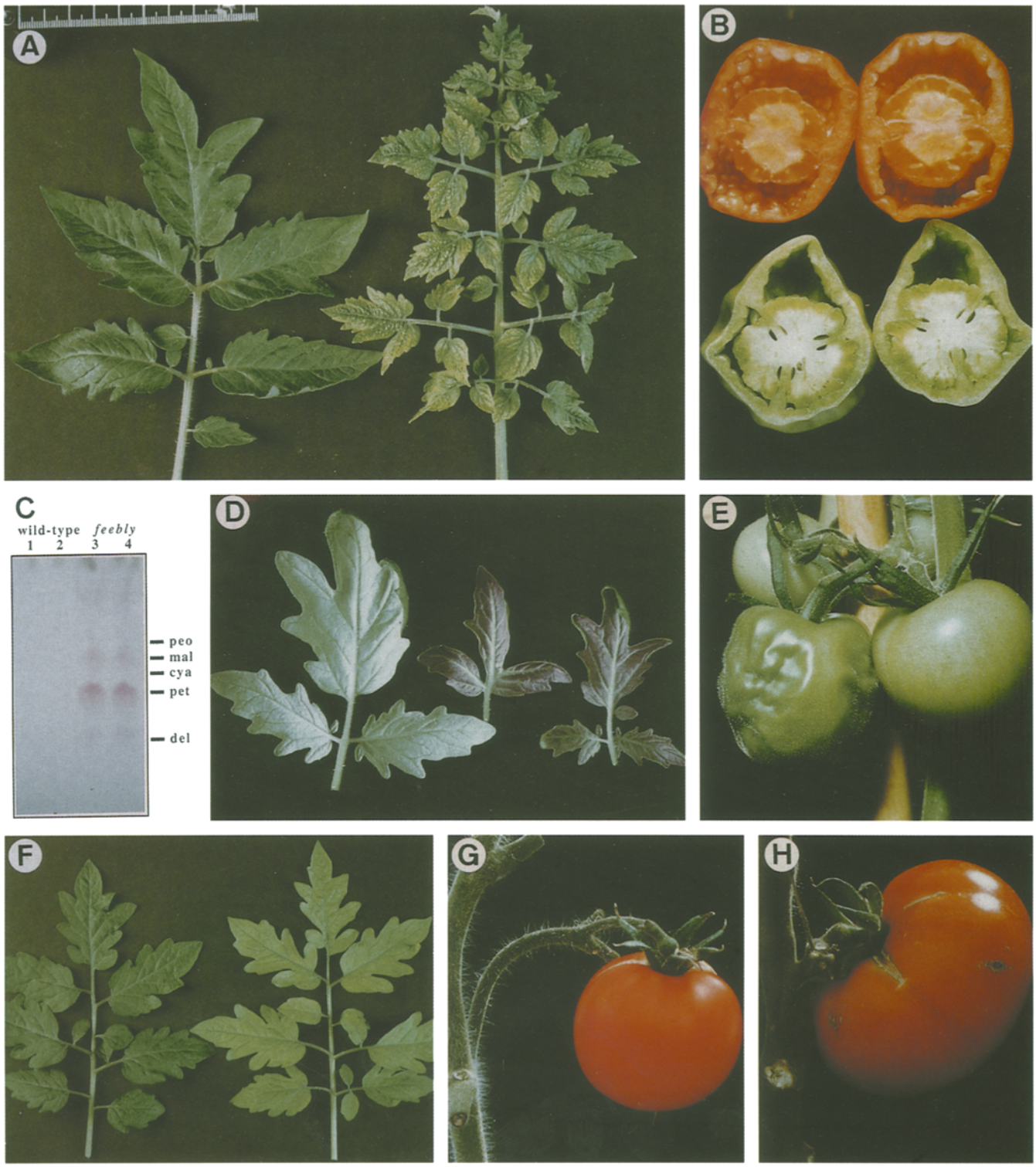


Fig. 3A–H Tomato mutants obtained following the transposon mutagenesis procedure. **A** Part of the upper side of a wild-type branch with five leaflets (*left*) and a complete branch of the *feebly* (*fb*) mutant (*right*). **B** Cross sections of fruits of the *fb* plants with empty locules. **C** Analysis of pigment production in first leaves of wild-type plants and in those of *fb* plants by thin layer chromatography (*cya* cyanidin, *del* delphinidin, *mal* malvidin, *peo* peonidin, *pet* petunidin). **D** First leaves of a wild-type plant (*left*) and of two *fb* plants (*right*) showing increased production of anthocyanins. **E** Irregular skin and unusual angular form of some *fb* fruits. **F** Leaves of a wild-type plant (*left*) and a *yellow jim* (*yj*) mutant (*right*). **G** Wild-type tomato pedicel and fruit. **H** Fruit of the *dopey* (*dp*) mutant with a strongly reduced pedicel

Seedlings with the *feebly* (*fb*) mutation appeared normal until the first leaves developed; these became dark purple in color on their undersides as a result of the appearance of anthocyanins. Spectrophotometric analysis of first-leaf extracts showed that the *fb* mutants produced on average tenfold higher anthocyanin levels than wild-type plants (data not shown). Thin layer chromatography showed that the anthocyanin accumulation consisted mainly of petunidin and, to a lesser extent, malvidin and delphinidin (Fig. 3). During development of the second leaves, the anthocyanins gradually disappeared. Further development of the *fb* plants was strongly retarded. The mutants exhibited slow and weak growth and had small, yellowish/pale-green, crinkled leaves. The habitus of mature *fb* plants was small and fragile; flowers appeared normal but fruit production was poor and seedset was very low. About one-third of the fruits developed irregular skins and unusual angular forms; ripening of these fruits was apparently normal but the locules did not contain gel and seeds were absent.

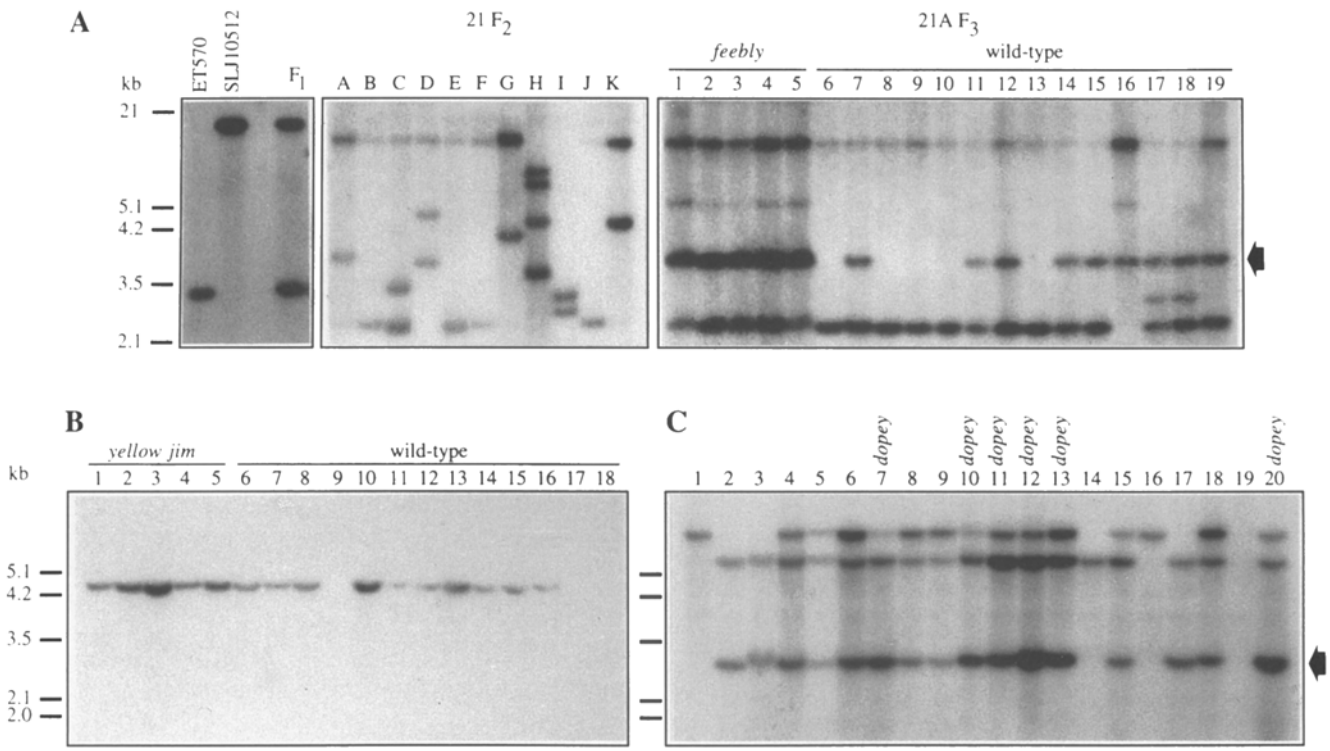
In contrast to the dark-green cotyledons and leaves of wild-type tomatoes, those of *yellow jim* (*yj*) mutants were yellow to light green; this was most obvious in immature leaf tissues (Fig. 3). Spectrophotometric analysis showed that the *yj* plants contained on average 50% of the chlorophyll levels of wild-type plants (data not shown). The reduced amounts of chlorophyll appeared not to affect the vigor or the fertility of the mutants. The *dopey* (*dp*) mutants showed retarded development and mature plants were smaller than wild-type plants. Most characteristic of the *dp* phenotype was the complete or partial absence of an inflorescence. In the latter case, the stalk connecting the inflorescence to the main stem of the plant was absent and these mutants also lacked the pedicels attaching the flowers to the main stalk of the inflorescence. As a consequence, the flowers were positioned close to each other in an unusually formed inflorescence. The fruits developed apparently normally, but fruit expansion was often hindered as a result of the reduced space available and the fruit, therefore, remained small.

Genetic linkage between mutant loci, transposed *Ds* elements and the *Ds* donor site

To analyze whether the *fb*, *yj*, and *dp* loci cosegregated with specific *trDs* elements, 40 plants of each F_3 progeny were molecularly analyzed. The mutant genotypes of all these F_3 plants (3×40) were determined by screening F_4 progenies ($n = 28$) for segregation of the particular mutation (*fb*, *yj*, *dp*). Southern analysis showed that the *fb* and *dp* segregants contained three different *trDs* elements, and that those of *yj* contained a single *trDs* (Fig. 4). In each of the three F_3 populations (*fb*, *yj*, *dp*), the presence of one distinct *Ds* insertion segregated with the mutation, i.e., the specific *trDs* was only present in plants that were homozygous (mutants) or heterozygous for the mutation. No recombinations between the *Ds* elements and the corresponding mutant loci were detected in the 40 plants of each F_3 progeny, indicating that the *fb*, *yj*, and *dp* loci were genetically closely linked (0.0–2.6 cM) to specific *Ds* elements.

The *Ds* element that cosegregated with the *fb* locus was also genetically mapped relative to the phenotypic marker *solanifolia* (*sf*) on the long arm of chromosome 3 by RFLP analysis. Part of the plant DNA that flanked the *Ds* element at the 3′ side was isolated by IPCR (FE3) and used as a hybridization probe. An interspecific F_2 population that segregated for *sf* and that permitted RFLP analysis was derived from *L. esculentum* Tester-3 and the wild tomato *L. pennellii* (Van der Biezen et al. 1995). To identify RFLPs, parental DNA digested with several restriction enzymes was hybridized with the FE3 probe (data not shown). Most enzymes showed distinct single restriction fragments, and *Eco*RV, *Hin*dIII and *Xba*I showed RFLPs between the two parents. Subsequently, DNA of plants homozygous for the *L. esculentum* allele (*sf/sf*) was digested with *Xba*I and hybridized with the FE3 probe. Out of 32 interspecific F_2 plants, 6 showed both parental restriction fragments as a result of recombination between *sf* and the *Ds* insertion site (data not shown). These results indicated a genetic distance between *sf* and the *Ds* element, and thus the *fb* locus, of 10.4 ± 3.8 cM.

To determine the genetic linkage between the mutant loci (*fb*, *yj*, *dp*) and the donor site (the ET570 T-DNA), recombinations were scored in the three F_3 populations, each consisting of 40 plants. The T-DNA genotypes of the F_3 plants were determined by testing the F_4 progenies ($n = 28$) for the presence of the *BAR* gene by spraying with 50 mg/l PPT. The F_3 plants segregating for the *dp* mutation were homozygous for the T-DNA (*BAR*) which precluded the linkage analysis. Interestingly, the *fb* mutants that lacked the *BAR* gene were not affected by PPT. The mechanism underlying the PPT insensitivity of these *fb* mutants is unknown, and this remarkable, unanticipated observation hampered the analysis of the T-DNA (*BAR*) segregation. Therefore, the presence of the T-DNA in the *fb* mutants was determined by PCR. Amplification by the PCR with the *35s/bar* primers (Fig. 1) with DNA from *fb* plants carrying the T-DNA generated DNA fragments of 0.8 kb, while no products were obtained with DNA of plants without the T-DNA (data not shown). The determination of the mutant genotypes (*fb* or *yj*) and the presence of the donor site T-DNA (*BAR*) permitted calculation of the recombinant fraction and, hence, the genetic distance (cM). The *fb* locus was shown to be tightly linked to the T-DNA (7.1 ± 4.1 cM), and the *yj* locus was mapped at a distance of 20.4 ± 6.2 cM from the T-DNA. Although *yj* has only been mapped relative to the T-DNA, its location was presumed to be



proximal because this ET570 T-DNA is one of the most distal markers on the long arm of chromosome 3 (Van der Biezen et al. 1994a). Finally, the linkage data of the *Ds* insertions, the *fb* locus, the *yj* locus, the T-DNA insertion and the *sf* locus (this study), and that of the same T-DNA and *sf* described previously (Van der Biezen et al. 1994a), were used to construct an integrated genetic map of the distal end of chromosome 3L with the computer package JoinMap (Fig. 5).

Analysis of *feebly* mutants for *Ac*-induced reversions to wild type

We then investigated whether the closely linked *Ds* element was inserted into a gene, thereby inactivating it and resulting in the *fb* phenotype. To transactivate the *Ds* element, the *fb* mutant was backcrossed with the *sAc*-containing line. The resulting BC_1 progenies (heterozygous for the *fb* mutation, and hemizygous for the *Ds* element, the *sAc* element and the T-DNA) were self-pollinated for the production of BC_1S_1 families. Following excision of the *Ds* element from the potentially inactivated *FB* gene, the BC_1S_1 progenies should reveal both somatic reversions and germinally transmitted reversions. Somatic reversion would result in unstable mutations that could appear as chimeric phenotypes, i.e., wild-type revertant sectors on mutant backgrounds. Germinally transmitted reversions would result in stable wild-type phenotypes in the BC_1S_1.

Fig. 4A–C Cosegregation of *Ds* elements with the mutant phenotypes: *fb*, *yj*, and *dp*. Southern analysis of *Eco*RI-digested DNA, hybridized with the radiolabeled AC3 probe, showed *sAc*- and *Ds*-specific restriction fragments. **A** *Left panel* The parental SLJ10512 (*sAc*) and ET570 (*Ds*) lines were crossed to produce doubly hemizygous F_1 plants (*sAc/Ds*). *Middle panel* Eleven (*lanes A–K*) Hyg/PPT-resistant, GUS^- siblings of F_2 family 21 that carried both unique and common, stable, germinally transmitted *Ds* transpositions. *Right panel* Cosegregation of the *fb* locus with an ~3.8 kb *Ds*-specific fragment (indicated by the *arrow*) in the self progeny of F_2 plant 21A ($n = 40$, 19 plants shown). *Lanes 1–5*: mutants (*fb/fb*); *lanes 7, 11, 12, and 14–9*: heterozygotes (*Fb/fb*); remaining lanes: homozygotes (*Fb/Fb*). **B** Cosegregation of the *yj* locus and an ~4.6 kb *Ds*-specific fragment in the F_3 ($n = 40$, 18 plants shown). *Lanes 1–5*: mutants (*yj/yj*); *lanes 6–8 and 10–16*: heterozygotes (*Yj/yj*); remaining lanes: homozygotes (*Yj/Yj*). **C** Cosegregation of the *dp* locus and an ~2.8 kb *Ds*-specific fragment (indicated by the *arrow*) in the F_3 ($n = 40$, 20 plants shown). *Lanes 7, 10–13, and 20*: mutants (*dp/dp*); *lanes 2–6, 8, 9, 15, 17, and 18*: heterozygotes (*Dp/dp*); *remaining lanes*: homozygotes (*Dp/Dp*)

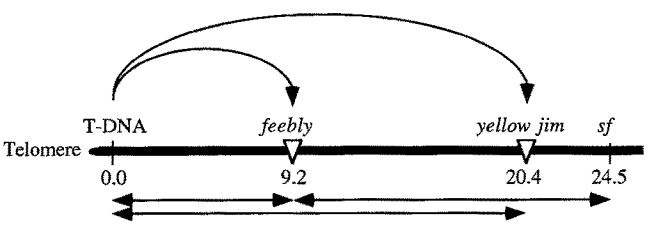


Fig. 5 Integrated genetic linkage map of the end of the long arm of chromosome 3 containing the ET570 T-DNA and the loci *fb* and *yj*. The mutant loci were mapped (indicated by the *arrows below the chromosome*) relative to the T-DNA, which is the most distal marker on chromosome 3L (Van der Biezen et al. 1994a); in addition *fb* was mapped by restriction fragment length polymorphism (RFLP) relative to *solanifolia* (*sf*). The *arrows above the chromosome* denote transpositions of *Ds* (*triangles*) from the T-DNA. Genetic distances are presented in centiMorgans

Table 2 Germinal reversion of *fb*

Family[a]	Total	*feebly*			Germinal excision frequency %[b]	ET570 T-DNA[c]			SLJ10512 T-DNA[d]		
		Wild-type	*fb*	[%]		PPT^R	PPT^S	%	GUS^+	GUS^-	%
Control	301	227	74	24.6	0	225	76	26.0	–	–	
FR-1	233	229	4	1.7	73	178	55	23.6	8	2	
FR-2	124	121	3	2.4	61	92	32	25.8	7	3	
FR-3	198	193	5	2.5	60	144	54	27.3	7	3	
FR-4	105	104	1	1.0	85	79	26	24.8	8	2	
FR-5	128	127	1	0.8	88	100	28	21.9	8	2	
FR-6	124	122	2	1.6	74	91	33	26.6	13	2	
FR-7	126	118	8	6.3	0	88	38	30.2	8	2	
FR-8	142	139	3	2.1	66	108	34	23.9	7	3	
FR-9	137	130	7	5.1	18	110	27	19.7	8	2	
FR-10	101	101	0	0.0	100	75	26	25.7	6	4	
FR-11	139	136	3	2.2	66	106	33	23.7	7	3	
FR-12	140	138	2	1.4	77	108	32	22.9	8	2	
FR-13	124	122	2	1.6	74	100	24	19.4	8	2	
FR-14	132	131	1	0.8	88	95	37	28.0	7	3	
FR-15	84	82	2	2.4	62	65	19	22.6	7	3	
FR-16	132	125	7	5.3	15	101	31	23.5	9	1	
FR-17	96	90	6	6.3	0	75	21	21.9	8	2	
Overall	2265	2208	57	2.5%	60	1715	550	24.3	126	41	24.6

[a] The FR progenies were derived by backcrossing the *fb* mutants with the *sAc* line and subsequent self pollination. As a control the *fb* mutant was outcrossed to the nontransgenic Moneymaker line and the OC_1 was self pollinated to produce OC_1S_1 progenies

[b] Germinal excision frequencies were calculated by estimating the deficit proportion on stable *fb* mutants (without *sAc*) from the statistically expected proportion (1/16). All the observed *fb* mutants lacked the *sAc* element

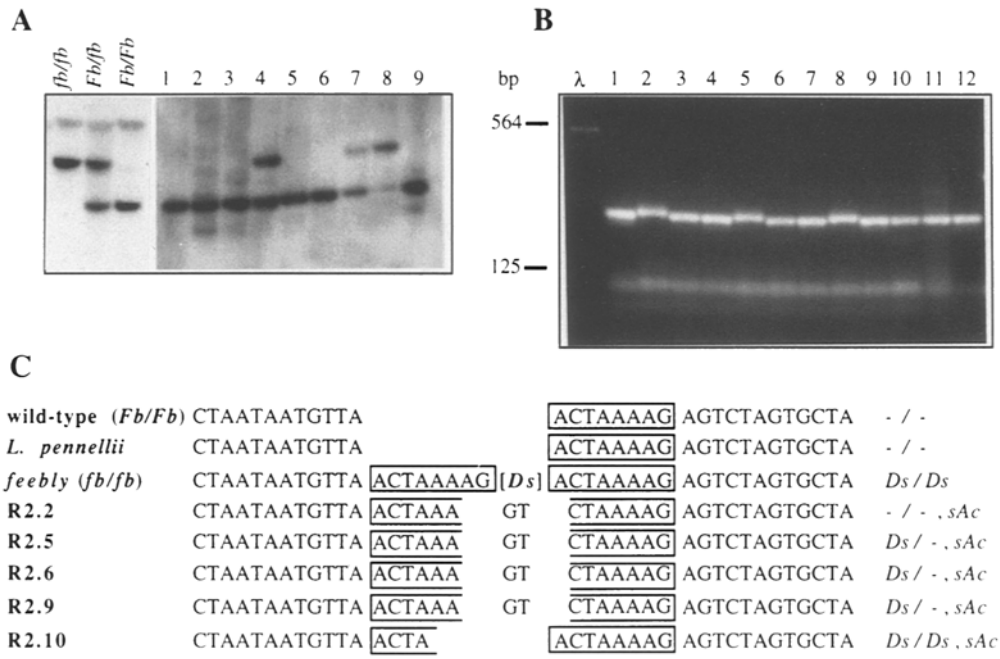
[c] The presence of the ET570 T-DNA (*BAR*) was scored by spraying the plants with 50 mg/l PPT; PPT^R resistant, PPT^S sensitive

[d] The presence of the SLJ10512 T-DNA (*GUS*) was scored by analysis of GUS activity (only wild-type plants included)

The low number of stable *fb* mutants (2.5%) in the BC_1S_1 progenies indicated *sAc*-induced unstable inheritance of the mutation, i.e., germinally transmitted reversions (Table 2). Somatically unstable *fb* mutants were not observed, suggesting that the *sAc* element also induced somatic excisions of *Ds* from the (non-cell-autonomous) *FEEBLY* (*FB*) gene leading to wild-type revertants. This suggestion was corroborated by the observation that none of the 57 stable *fb* mutants contained the *sAc* element as determined by GUS analysis and PCR with the *ac6/ac7* primers. It would be expected statistically that 1/16 of the total number of BC_1S_1plants ($n = 2265$) were *sAc*-lacking *fb* mutants, i.e., 142 plants instead of 57. The absence of 85 plants (60%) from this class (*fb/fb*, sAc^-) indicated an average frequency of germinal *Ds* excisions from the *FB* gene of 60%, resulting in germinally transmitted revertant *Fb* alleles (Table 2). This calculation of the germinal excision frequency relied on Mendelian 3:1 segregation ratios of the *fb*:*Ds* allele and the *sAc* T-DNA. Analysis of PPT resistance (*BAR*) and GUS activity of the same BC_1S_1plants showed that the *sAc* T-DNA (SLJ10512) and the *Ds* donor site T-DNA (ET570), which was closely linked to the *fb* locus, both segregated normally (Table 2). Furthermore, in control crosses without the *sAc* element the expected proportion (1/4) of *fb* mutants was observed (24.6%), indicating Mendelian transmission of the *fb*:*Ds* allele.

The *fb* genotypes of the wild-type BC_1S_1plants were determined by Southern analysis using the plant DNA sequence flanking the *Ds* insertion at the 3′ end (FE3) as hybridization probe. All three genotypes were detected: (1) plants that did not contain the *Ds* insertion allele (*Fb/Fb*), (2) plants that were heterozygous for the *Ds* insertion allele (*Fb/fb*) and, moreover, (3) wild-type plants were identified that were homozygous for the *Ds* insertion allele (*fb/fb*) but also contained a small proportion of the wild-type *Fb* allele (Fig. 6a). Southern analysis of these BC_1S_1 plants with the *Ds*-specific probe (AC3) showed that *Ds* was transpositionally active. The observation that the *fb/fb* plants contained a small proportion of cells with the wild-type *Fb* allele and also carried the *sAc* element, confirmed the conclusion that somatic excisions in these cells restored the wild-type phenotype, and were in agreement with the suggestion that the *FB* gene encodes a non-cell-autonomous factor.

Upon insertion of *Ac/Ds* family members, 8 bp target site sequences are duplicated and often a few central base pairs of the insertion site are modified following excision (Sutton et al. 1984). Based on the DNA sequence flanking both sites of the *Ds* insertion in the *fb* mutants, the *fe3* and *fe5* primers were designed to amplify the site of insertion by PCR (Fig. 8). No fragments were obtained with these primers using DNA of the stable *fb* mutants (sAc^-). However, the *act5b/*



fe5 primers generated fragments of the expected size (0.5 kb), confirming that all *fb* mutants were homozygous for the *Ds* insertion. Using the *fe3/fe5* primers in PCRs on DNA of 45 wild-type plants from three BC_1S_1 families, fragments of expected size (~0.25 kb) were amplified in all samples. The slightly larger size of 14 PCR products indicated the presence of target site duplications (Fig. 6b). DNA sequence analysis of seven PCR products indeed showed that the target site ACTAAAAG was duplicated in five larger PCR fragments and not in two smaller fragments (Fig. 6c). Although some plants could have been heterozygous for the *fb* reversion allele, no PCR mixtures were observed that consisted of both DNA fragment sizes. Within one BC_1S_1family, two distinct *Ds* footprints were detected. Three plants that were hemizygous for the *Ds* insertion and one plant without the *Ds* element shared the same excision sequence (Fig. 6C, R2.5–9). Most probably, the *Ds* footprint resulted from excision in the BC_1 generation and was germinally transmitted to several BC_1S_1 plants. These results were in line with the observation that germinal reversions occurred with high frequency (60%). The second type of excision allele (Fig. 6C, R2.10) was isolated from a wild-type plant homozygous for a somatically active *Ds* insertion (Fig. 6A, lane 8) and indicated that somatic reversions coincided with excision of *Ds* from the *FB* gene. Self progenies (BC_1S_1; $n = \sim 60$) of all five plants with the two types of excision alleles showed *fb* mutants with low frequencies (0–8%). The number of added base pairs in the revertant alleles (+7 bp and +4 bp) would not restore an open reading frame (ORF), suggesting that the *Ds* was inserted in a noncoding region of the *FB* gene.

Fig. 6A–C Molecular analysis of the *fb* mutant. **A** Determination of *Fb* genotypes of wild-type BC_1S_1 plants by Southern analysis of *Hin*dIII-digested DNA, hybridized with the radiolabeled FE3 probe. No *Hin*dIII recognition sites are present within the *Ds* element and, consequently, the restriction fragment of the *Ds* insertion allele was 5 kb larger than that of the wild-type allele. The first three lanes show control plants with defined genotypes: *fb/fb*, homozygous for the *Ds* insertion (mutant); *Fb/fb*, hemizygous for the *Ds* insertion; *Fb/Fb*, no *Ds* insertion. *Lanes 1–6, and 9*, no *Ds* insertion (*Fb/Fb*). *Lanes 4* and *7*, hemizygous for the *Ds* insertion (*Fb/fb*). *Lane 8*, homozygous for the *Ds* insertion (*fb/fb*). As a result of somatic excision of *Ds*, a small proportion of the DNA of this plant consisted of the wild-type *Fb* allele. All nine BC_1S_1 plants carried the *sAc* element. **B** Polymerase chain reaction (PCR) products generated with the *fe3* and *fe5* primers with DNA of BC_1S_1 plants, electrophoresed through an ethidium bromide-containing 2% agarose gel and visualized by UV light. The primers generated ~250 bp PCR fragments from DNA of control Fb alleles of wild-type tomato (*lane 11*) and *Lycopersicon pennellii* (*lane 12*). *Lanes 2, 5 and 8* contained slightly larger DNA fragments, which indicated that these plants carried *Fb* excision alleles with target site duplications. **C** DNA sequence analysis of *Ds* footprints following excision of *Ds* from the *FB* gene. The fragments were amplified by PCRs with the *fe3/fe5* primers. The 8 bp target site of *Ds* is *boxed*. The DNA sequences of the *Ds* insertion sites of wild-type tomato (*Fb/Fb*) and *L. pennellii* were identical; the *fb* mutant (*fb/fb*) had the *Ds* inserted between the target site and the duplication. Some BC_1S_1 plants (R2.2–R2.10) had slightly larger PCR products and showed remnants of the 8 bp target site and its duplication

Northern and cDNA analysis of *FEEBLY*

The two IPCR products FE3 and FE5 containing plant DNA sequences flanking each side of the *Ds* element in the *fb* mutant (GenBank Accession Number U35644), both hybridized to an approximately 1.6 kb transcript in total and poly(A)$^+$ RNA isolated from roots, stems

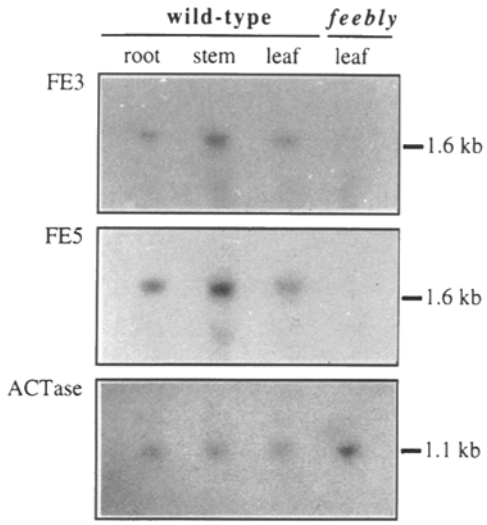


Fig. 7 Expression of *FEEBLY* in wild-type and *fb* plants. Northern analysis with total RNA from roots, stems and leaves of a wild-type plant and leaves of the *fb* mutant, hybridized with the radiolabeled inverse PCR (IPCR) products FE3 and FE5 that contain plant DNA sequences flanking the *Ds* element in the *fb* mutant; the tomato *ACTase* gene was used as control. The FE3 and FE5 probes both hybridized to a 1.6 kb RNA transcript of a wild-type plant but not with RNA of the *fb* mutant

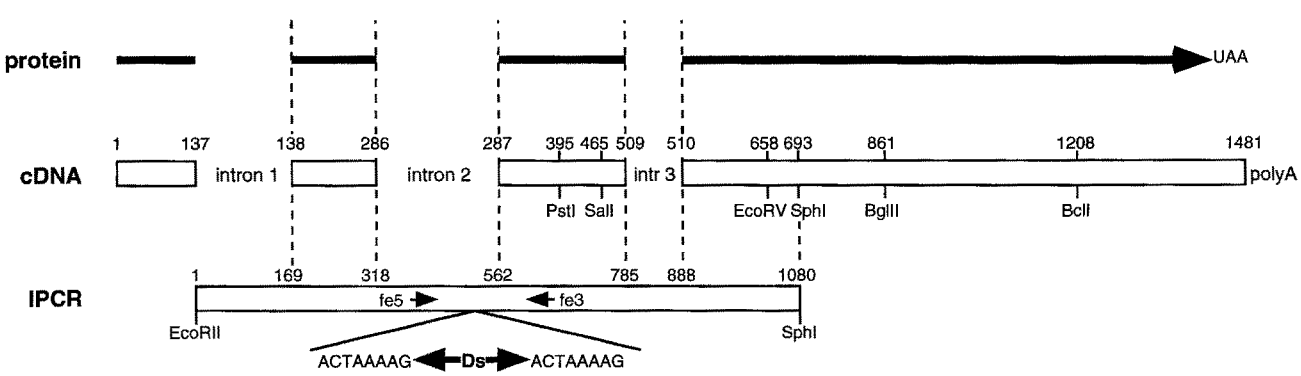
and leaves of nontransgenic wild-type plants (Fig. 7). These results indicated that the *FEEBLY* (*FB*) gene was transcribed at the whole plant level. No hybridizing *FB* transcripts were observed in total and poly(A)$^+$ RNA of leaves isolated from the *fb* mutants, indicating that the *Ds* insertion resulted in the absence of a steady-state RNA. Control hybridizations with the *BAR* gene and the tomato aspartate carbomoyltransferase (*ACTase*) gene (Overduin et al. 1993) as probes, showed RNA transcripts of expected sizes in the wild-type plants as well as in the *fb* mutants.

Hybridization of approximately 50000 cDNA λ clones with the radiolabeled FE3 and FE5 IPCR products, resulted in four positive clones that contained inserts of the expected sizes (~1.6 kb). Sequencing of one cDNA clone (GenBank accession U35643) showed that the transcript consisted of an ORF encoding 484 amino acids, and contained a poly(A) tail indicating the completeness of the 3′ end (Fig. 8). Although the size of the cDNA sequence corresponds to the size of the transcript detected by Northern analysis, it is not known whether the sequence is complete at the 5′ end; the first AUG translation start is located at position 120 of the ORF. Alignment of the cDNA sequence with the genomic sequences obtained by IPCR, showed the presence of three introns, in one of which *Ds* was inserted (Fig. 8). Comparison of the genomic and cDNA sequences with database sequences revealed no significant homology with other genes with known function, neither did it reveal any conspicuous motifs. Different parts (~0.2 kb) of the cDNA sequence showed high homology (~70% identity) with three anonymous partial 3′ cDNA clones from Arabidopsis (expressed sequence tags with GenBank Accession Numbers T46146, Z27292, and R30347).

Discussion

Using reporter genes to monitor *Ds* transpositions and the presence of an *sAc* element, 420 plants bearing stable, germinally transmitted *Ds* elements were selected. To reveal whether the *Ds* insertions were inserted in genes that upon inactivation result in deviant phenotypes, self progenies were screened for segregation of severe visible phenotypes. Three families were identified that segregated for single recessive mutations: *feebly* (*fb*), *yellow jim* (*yj*) and *dopey* (*dp*). Southern analysis showed that the three loci were genetically

Fig. 8 Molecular maps of the genomic (IPCR) and mRNA (cDNA) sequences of the *FB* gene. Some restriction enzyme sites are shown, as well as the position and direction of the *fe5* and *fe3* oligonucleotide primers used for PCR (*arrows*). The IPCR fragments flanking the *Ds* element were obtained following *Eco*RII (5′ *Ds*) and *Sph*I (3′ *Ds*) digestion, and were used to identify a cDNA clone. Both strands of all clones were sequenced. The DNA sequence of the cDNA consists of an open reading frame of 484 amino acids and is terminated by an ochre stop codon (UAA). Sequence alignments of the IPCR fragments with the cDNA clone revealed the presence of three introns, in to one of which *Ds* had been inserted



linked to distinct *Ds* insertions. In addition, segregation analysis showed that the *fb* and *yj* loci were linked to the donor site T-DNA. Since germinal *Ds* transpositions in tomato predominantly take place to linked sites (Carroll et al. 1995; Knapp et al. 1994), it is not unlikely that the mutations were induced by *Ds* transpositions. The resolution of linkage analysis is insufficient to determine whether the mutations were due to the insertion of the *Ds* elements or to footprints of *Ds* elements left behind following closely linked secondary transpositions. Therefore, reversion analysis was employed to determine whether excision of the *Ds* elements resulted in restoration of the wild-type phenotypes. Molecular genetic analysis indicated that *sAc*-induced excision of the *Ds* element restored the *fb* mutation; reversion analysis of the *yj* and *dp* mutants is in progress. To our knowledge the *dp* mutant has not been reported before; genetic complementation experiments are in progress to determine whether *fb* and *yj* are new alleles of previously described mutant loci with corresponding chromosomal positions: *divaricata* (*div*) and *aureata* (*aut*), respectively (Stevens and Rick 1986). The phenotypes and the chromosomal position of the *feebly* (*fb*) mutation and the previously described mutation *divaricata* (*div*) (Stevans and Rick 1986) are similar, which suggests that both genes are alleles at the same locus. Therefore, crosses were made with the *fb* (staminate parent) and *div* mutants to investigate whether the mutants can complement each other. Southern analysis (*Eco*RI/AC3 probe) demonstrated that the progenies were the result of a cross. Since all progenies showed identical phenotypes as that of both parents, no complementation was established, confirming that *fb* is allelic to *div*. Hence, the designation *FEEBLY* (*FB*) may be replaced by *DIVARICATA* (*DIV*).

It was estimated that in two generations 471 unique *Ds* insertions were produced. The germinal *Ds* transposition frequency (9.1%), the proportion of unique *Ds* insertions (82%) and the *Ds* copy number (1–5) were comparable to those observed from other T-DNA locations in tomato (Carroll et al. 1995; Knapp et al. 1994). The presence of several unique germinally transmitted *Ds* copies in some F_2 plants, indicated multiplication of *Ds* elements, as has previously been reported for *Ac* elements in tomato (Yoder 1990). Most probably, the high proportion of unique *Ds* insertions was the result of independent germ line transpositions late in the development of the F_1 plants. In addition, secondary transpositions might have contributed to this high proportion as well. Forty-one percent of the selected seedlings were homozygous for the *Ds* donor site T-DNA, which does not significantly deviate ($P > 0.05$) from the expected proportion in an F_2 (1/3). As a consequence, 13% of the resistant plants did not contain transposed *Ds* elements but were selected on the basis of the presence of an FDS allele (*HPT*) conferring Hyg resistance, and an EDS allele (*BAR*) conferring PPT resistance. Selection of plants that were homozygous for the *Ds* donor site T-DNA could have been avoided by outcrossing the F_1s to a nontransgenic line, resulting in progenies that are all hemizygous for the T-DNA.

The frequencies with which the three mutants (*fb*, *yj* and *dp*) were obtained were 7.1×10^{-3} of the total of 420 selected Hyg/PPT-resistant and GUS^- plants, and 6.4×10^{-3} of the estimated total of 471 unique *trDs* elements. These frequencies are in the same range as the frequencies of *Ac*/*Ds*-induced mutants in maize (Gierl and Saedler 1992), Arabidopsis (Bancroft et al. 1993; Long et al. 1993) and the frequencies statistically predicted in heterologous hosts (Van der Biezen et al. 1994b). Detailed phenotypic examination – irrespective of the mutant segregation ratios – resulted in the establishment of a higher mutant frequency (7.4×10^{-2}) in Arabidopsis (Altmann et al. 1995). However, out of the 31 analyzed mutants only 3 mutant loci cosegregated with a *Ds* insertion, indicating that the other mutations had occurred during the process of generating plants with transposed *Ds* elements. To identify mutants, we screened for Mendelian segregation of severe deviant phenotypes up to the 4–5 true leaves stage. These strict selection criteria imply that embryonic lethality, flowering, fertility and fruit mutants were missed and ensured that poorly represented or subtle mutant phenotypes were not selected for further analysis, i.e., mutant leaves that were abnormal in the number or subdivision of the segments, surface texture, marginal conformation, angle of emergence from the stem, venation patterns, etc (Stevens and Rick 1986). Consequently, it is conceivable that a considerable proportion of mutants were unnoticed and hence, the mutant frequencies would be underestimates. Starting with a single *Ds* donor site T-DNA on chromosome 3L, we generated tomato plants carrying germinally transmitted, transposed *Ds* elements. Because transposition predominantly takes place to genetically linked sites, the mutagenic potential of a particular *Ds* line is dependent on the density of genes in the vicinity of the T-DNA that upon inactivation result in recognizable phenotypes. Therefore, additional *Ds* lines should be used to determine reliably the frequency and spectrum of transposon-induced mutants in tomato.

The insertion of the 5 kb *Ds* element in an intron of the *FEEBLY* (*FB*) gene resulted in a loss-of-function mutation. RFLP analysis showed that the genomes of tomato and its wild relative *L. pennellii* each contain a single copy of the *FB* gene on chromosome 3. Northern analysis indicated that *FB* is constitutively expressed at moderate levels. Heterozygotes (*Fb*/*fb*) were normal and the transmission of the *Ds* insertion alleles followed Mendelian frequencies. The weakly growing *fb* mutants produced high anthocyanin levels during seedling development and, subsequently, slowly developed into fragile plants with small yellowish to pale-green leaves and some deformed fruits. Fertility of the mutants was reduced but viable seeds could be obtained. Excisions of *Ds* from the *FB* gene resulted in

reestablishment of the wild-type phenotype. With regard to the footprints of *Ds* after excision from the *FB* gene, the insertion in the noncoding region allowed the somatic and germinal reversions to occur with high frequencies (100% and 60%, respectively). Molecular analysis indicated that a small proportion of cells with the wild-type *Fb* allele were able to rescue the mutant phenotype and suggested that the FB protein has a non-cell-autonomous effect.

The FB protein is most likely to be important for vigorous plant development. The observation that the accumulation of anthocyanins and the development of deformed fruits can both be induced in tomato by environmental factors indicates that these features might be pleiotropic. The increased anthocyanin levels may result from physiological stress conditions such as nutrient deficiency (nitrogen, phosphorus, sulfur), or defective regulation of growth factors (hormones, light) (Salisbury and Ross 1992). In addition, the large variation in anthocyanin concentrations between the *fb* mutants suggested that the production of the pigments was a secondary effect. Furthermore, the tomato disease "puffiness" shows identical fruit symptoms (poor pollination, poor gel formation and angular appearance) and results from environmental factors: low or high temperatures, conditions of drought or excessive water, or excessive nitrogen fertilization (Stevens and Rick 1986; Watterson 1985). Possibly, FB is involved in a metabolic pathway and the absence of the protein primarily results in physiologically disturbed plants that show pleiotropic effects. The observation that the *fb* mutants lacking the *BAR* gene were nevertheless insensitive to PPT might represent a valuable clue to the role of FB. PPT inhibits the biosynthetic enzyme glutamine synthase (GS), which uses ammonia as one of its substrates (De Block et al. 1987). The *BAR* gene encodes PPT acetyltransferase (PAT), which detoxifies PPT. Sensitivity to PPT is caused by buildup of lethal ammonia levels. Although, it cannot be excluded that the *fb* plants are affected in the uptake or the transport of PPT, it is possible that endogenous ammonia levels cannot attain lethal concentrations in the mutants and, therefore, the *fb* plants were not affected by PPT. Hence, the FB protein might be involved in nitrogen metabolism; the absence of FB would lead to disturbed levels of intermediates of the nitrogen pathway, resulting in PPT insensitivity, retarded growth, high anthocyanin production and deformed fruits.

Transposon tagging using the *Ac/Ds* system from maize in heterologous hosts has proven its value as an approach to the isolation of genes with unknown products. The establishment of transgenic tomato lines with transposons at mapped genomic locations (Knapp et al. 1994; Thomas et al. 1994), permits targeted (Jones et al. 1994) and nontargeted (this study) transposon tagging experiments. In principle, no molecular analysis is required for the induction of mutants and for the verification of the presence of transposon insertions. Using straightforward techniques, the corresponding genes can be cloned and characterized. We recovered three mutant phenotypes of which the *fb* mutation was investigated in detail. Only *fb* mutants that carried stable *Ds* insertions were obtained; in the presence of the *sAc* element somatic excisions restored the wild-type phenotype. These results indicated that the *fb* mutant would not have been recovered if the autonomous *Ac* element had been used as mutagen. Therefore, it is suggested that insertional mutagenesis with stable nonautonomous transposons leads to a wider mutational spectrum.

Acknowledgements Klaus Theres is acknowledged for the tomato line containing the *Ds* element (ET570), Jonathan Jones is thanked for the *sAc* line (SLJ10512), and Jan van Kan for the cDNA library. Donato Giannino and Jeanine Robles are acknowledged for technical assistance. This project was financially supported in part by the BRIDGE program of the European Community (BIOT-900192).

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