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Insertional mutagenesis in *Arabidopsis thaliana*: isolation of a T-DNA-linked mutation that alters leaf morphology

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Summary. We investigated the potential of the *Agrobacterium tumefaciens* T-DNA as an insertional mutagen in *Arabidopsis thaliana*. *Arabidopsis* lines transformed with different T-DNA vectors were generated using a leaf disc infection procedure adapted for efficient selection on either kanamycin or hygromycin medium. A standardized screening procedure was developed for the detection of recessive mutations in T2 populations of regenerated and/or transformed lines. Recessive mutations originating from the tissue culture procedure occurred at a low frequency – between 2% and 5%. Within 110 transformed lines that contained a total of about 150 T-DNA inserts, one recessive mutation, named *pfl*, cosegregated with a specific T-DNA copy. This *pfl* mutation mainly affected the morphology of the first seedling leaves under normal growth conditions and was mapped to chromosome 1. No recombination between the *pfl* locus and the kanamycin resistance marker on the T-DNA was detected when screening F2 and F3 populations of a mutant crossed to the wild type. The maximal genetic distance between the *pfl* locus and the kanamycin resistance gene, determined as 0.4 ± 0.4 cMorgan, strongly suggests that the *pfl* mutation is induced by the insertion of the T-DNA. Our finding of one T-DNA-linked recessive mutation in 110 transgenic lines indicates that T-DNA can be used for mutagenization of the *Arabidopsis* genome under tissue culture conditions.

Key words: *Agrobacterium tumefaciens* – Insertional mutagenesis – Linkage analysis – Selectable marker genes – Transformation

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Introduction

The molecular regulation or triggering of plant-specific developmental pathways is poorly understood due to the lack of cloned regulatory genes. Since the gene products involved in these processes are unkown, molecular gene cloning techniques based on mRNA or protein purification are not possible. For this reason insertional mutagenesis could be a powerful tool to isolate genes involved in critical steps in plant morphogenesis. The insertion of a well-characterized DNA sequence into a gene of interest allows the mutated and wild-type genes to be cloned by standard techniques. Developmentally important genes have been identified in mammals and *Drosophila* using insertion sequences as mutagens (Bingham et al. 1981; Gridley et al. 1987). In plants, this approach has been used to clone a limited number of genes in species that have well-characterized endogenous transposable elements (Shepherd 1988).

The plant species *Arabidopsis thaliana* contains a small genome with little repetitive DNA and small gene families that make it an attractive model system for molecular analysis (Meyerowitz 1987). Moreover, this plant is ideally suited for genetic mutation analysis since it is short-cycling, small, and self-fertilizing (Koornneef 1987; Haughn and Somerville 1988). Because of its characteristics, this species can be considered as a model system to study the potential of insertional mutagenesis in plants. In this paper, we studied the mutagenic capacity of the *Agrobacterium tumefaciens* T-DNA in *Arabidopsis*. T-DNA has been developed into an efficient vector for the introduction of genes into plants. The engineered T-DNAs are stably integrated in the plant genome, at low copy number and supposedly at random distribution (Weising et al. 1988; Gheysen et al. 1989). Although much is known about the basic process of T-DNA trans-

fer from *Agrobacterium* to the plant cell, little is known about the integration preferences in the plant DNA (Zambryski 1988). T-DNA can integrate into transcribed regions of the plant genome (André et al. 1986; Koncz et al. 1989), and its insertion can lead to the disruption of gene activity (Marks and Feldmann 1989; Herman and Marks 1989; Koncz et al. 1990).

We estimated the frequency with which T-DNA-linked mutations occur in transgenic *Arabidopsis* lines obtained with a leaf disc infection procedure making use of several, different selectable marker genes to follow T-DNA in the genetic analyses. One mutation was found that showed tight genetic linkage to a T-DNA insert. This mutation was subsequently mapped and its specific changes in leaf morphology was determined on a mathematical basis. The T-DNA mutagenesis frequency obtained in our transformation system is compared to the ones found by Feldmann et al. (1989) and Koncz et al. (1990), and is discussed.

Materials and methods

Bacterial strains

Chimeric genes contained between the left and right T-DNA borders were introduced in the *Agrobacterium tumefaciens* C58ClRif^R (pGV2260) strain and cointegrated in the T-DNA-less pTi derivative pGV2260 (Deblaere et al. 1985). pGSATE1 contains the $PT_R 2'$-*neo* and $PT_R 1'$-*hpt* selectable marker genes coding for kanamycin and hygromycin resistance, respectively (Vandekerckhove et al. 1989); pGSFR1161 (Valvekens et al. 1988) contains the $PT_R 1'$-*neo* chimeric gene and $PT_R 2'$-*bar* conferring resistance for the herbicide Basta® (Hoechst) to plants (De Block et al. 1987); pGV2260::pGVp246 (P. Breyne, unpublished data) contains a hygromycin resistance marker and the T-DNA gene 2-negative selectable marker (Budar et al. 1986). Bacterial cultures were grown in Luria Broth with shaking at 200 rpm.

Plant material, media, and culture conditions

Arabidopsis thaliana collection number C24 was used throughout all experiments and was provided by M. Jacobs (Vrije Universiteit Brussel, Brussels, Belgium). The marker line W100, provided by M. Koornneef, contains two genetic markers on each of the five chromosomes (Koornneef et al. 1986).

The different plant media were: (1) germination medium (GM) [Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog 1962), 10 g/l sucrose]; in minimal GM vitamins are omitted, and enriched GM is supplemented with vitamins, nucleic acids, and amino acids as described by Baus et al. (1986); (2) C1 [MS salts, B5 vitamins (Gamborg et al. 1968), 30 g/l sucrose, 0.1 mg/l naphtalene acetic acid (NAA), 1 mg/l 6-benzyl-aminopurine (BAP)]; (3) C2 [C1 medium supplemented with 0.5 g/l polyvinylpyrrolidone, $M_r = 10{,}000$]. All plant media were buffered with 0.5 g/l of 2-(*N*-morpholino) ethane sulfonic acid to a pH 5.7 (1 *M* KOH) and solidified with 8 g/l Difco Bacto agar.

Arabidopsis seed sterilization and growth-room conditions for tissue culture were as described by Valvekens et al. (1988). *Arabidopsis* plants were grown under nonsterile conditions in a conditioned growth chamber under a 16-h day/8-h night cycle at 22 °C, 160 μmol/m^2 per sec light intensity, and at 75% relative humidity.

Arabidopsis leaf disc infection method

The leaf disc infection of Lloyd et al. (1986) was optimized. Rosette leaves of 3-week-old, sterile seedlings, germinated as described, were cut transversely into two pieces and incubated for 4 days on C1 medium prior to infection. The leaf pieces were then transferred to 9 ml liquid C1 medium containing 1 ml of an overnight *Agrobacterium* culture and incubated for 5–10 min. The infected leaf pieces were blotted on sterile filter paper and incubated on solidified C1 medium for 48 h. After this incubation period, the bacteria had slightly overgrown the leaf discs. The leaf discs were washed in liquid C1 medium containing 500 mg/l Claforan® (Hoechst), blotted onto sterile filter paper, and transferred to selective C2 medium containing 500 mg/l Claforan® and 50 mg/l kanamycin sulfate (Sigma) or 20 mg/l hygromycin (Calbiochem). After 2 weeks of incubation on the selective C2 medium, transformed green callus grew along the cut surface of the infected leaves whereas the negative control showed no callus growth or very small, yellow-brownish callus. After 4 weeks of selection, nicely expanded shoots were removed and transferred to GM medium for rooting. Simultaneously, little pieces of callus with short shoots were transferred to GM medium to allow the small shoots to expand. After 1 week the expanded shoots were cleared from all callus tissue and put onto large petri dishes (150 × 25 mm) with fresh GM medium for rooting. Two to three weeks later, rooted shoots (T1 shoots) were either transferred to soil for seed set or allowed to form seeds in petri dishes (Valvekens et al. 1988). About three and one-half month after infection, ripe seeds (T2) were harvested from plants grown in petri dishes or in soil. Seeds were dried for 2–4 days at 28 °C and then vernalized for 5–7 days at 7 °C before they were used for genetic analysis. The Claforan® concentration was gradually lowered to 400 mg/l after 2 weeks of selection, to 250 mg/l 2 weeks later, and omitted after another 2 weeks. Kanamycin or hygromycin selection is maintained for at least 6 weeks to avoid the formation of nontransformed shoots.

Determination of ploidy level

The ploidy level of leaf disc-derived lines was determined by chloroplast counting of the epidermal guard cells and confirmed for a few predicted diploid and tetraploid lines by propiono carmine staining of metaphase-blocked chromosomes in root tip squashes as described by De Block (1988), with the exception that the hydrolysis was done for 7 min in 1 *N* HCl at 60 °C.

Genetic mutation analysis

Population sizes of 60 to 100 T2 seedlings were screened per transformed or untransformed regenerant (T1) for the presence of a recessive mutation segregating in a 1:3 ratio to wild-type siblings. The seedlings were germinated at a density of 32 per 25 × 150 mm petri dish (Falcon) containing minimal GM medium and grown for 4 weeks under sterile conditions. The plantlets were subsequently transferred to soil in separate small containers in a conditioned growth chamber for screening the adult phase of the plant. This procedure allowed the detection of alterations in the germination process, autotrophic growth, morphology, flower development, flowering time, and embryogenesis. The mutations detected in the T2 populations were confirmed by analyzing the T3 populations of the T2 seedlings that were homozygous or heterozygous for the respective mutations.

Genetic tests for marker gene expression

The germination of transformed T2 seed populations on medium containing kanamycin (50 mg/l) or hygromycin (20 mg/l) enabled us to distinguish between growing resistant plants and

dying sensitive plants after 2 weeks. At this stage the hygromycin-sensitive seedlings could be rescued on nonselective GM and restored to the wild type in about 5 days; kanamycin-sensitive seedlings could not. The germination of gene 2-transformed T2 populations on GM supplemented with 1 mg/l naphthalene acetamide (NAM) resulted in the segregation of malformed and arrested seedlings expressing gene 2 and wild-type-looking individuals devoid of gene 2. A clear interpretation of this germination assay on NAM is only possible if the seedlings are grown at low density (10–20 seedlings per 9-cm diameter petri dishes).

Callus induction tests were done on expanded cotyledons or leaf pieces of 2- to 5-week-old sterile plantlets. They were incubated with lower epidermis down on C1 medium containing 50 mg/l Km or 20 mg/l Hyg. After 2–3 weeks resistant leaves form large, green, shooting calli at the cut surface, whereas sensitive leaves do not form callus at all or very small, yellow-brownish, dying calli.

Basta® (Hoechst) spray tests were essentially done as described by De Block et al. (1987). Herbicide treatment was most effective on greenhouse-grown *Arabidopsis* plants in the vegetative phase with expanding rosette leaves, before bolting starts. Application of a 2 l/ha dose killed wild-type plants within 10 days. Herbicide-resistant plants tolerate doses up to 8 l/ha without damage.

Southern analysis

Plant DNA preparation, DNA gel blot analysis, and determination of T-DNA copy number was essentially done as described by Valvekens et al. (1988).

To determine the cosegregation of a T-DNA insert to a mutation, DNA was prepared from several individual T2 mutant plants or pooled T3 mutant populations, grown nonselectively, EcoRI digested (no cut whithin T-DNA), and hybridized to a T-DNA probe. In this way the segregation pattern of each T-DNA insert was visualized in every mutant individual.

Leaf shape determination

The perimeter of the first two leaves of 3-week-old wild types or mutant seedlings, grown aseptically or under nonsterile conditions, was magnified by using a microscope with drawing tube at 50× magnification. The magnified figures were taken to determine length and width values.

Results

Leaf disc transformation using kanamycin or hygromycin selection

An efficient leaf disc infection method was developed for *Arabidopsis* C24 (Materials and methods). Several parameters greatly influenced the transformation efficiency. We found that a 4-day hormone treatment of the leaf discs prior to infection was a crucial step in obtaining high transformation efficiencies. Transformation efficiencies increased from less than 1% (with or without a 2-day preculture) to as high as 50% when a 4-day preculture was used. Longer hormone pretreatments (6 days) resulted in the proliferation of untransformed tissue. Shoot regeneration was improved by using young expanding leaves as starting material, by subculturing tissues every 10–14 days, by using gas-diffusible tape to seal the petri dishes, and by reducing the Claforan® concentration as described (Materials and methods) (data not shown). The use of feeder layers during co-cultivation did not affect transformation efficiencies (data not shown).

With this optimized procedure, we tested the kanamycin versus hygromycin selection after transformation with the *Agrobacterium* strain pGSATE1 (Materials and methods). On each of the selective agents 50% of the infected leaf discs produced transformed fertile shoots, and transformed seeds could be harvested 3 to 3.5 months after infection. Similar transformation efficiencies of between 50% and 70% were obtained with *Agrobacterium* strains containing other T-DNA constructs with the P*nos* or PT_R promoter controlling the *hpt* or *neo* gene (data not shown). We estimated that on average 5% of the transgenic lines were tetraploids (Materials and methods) and that 5% to 10% of the transgenic lines did not show any or only a very weak resistance to hygromycin or kanamycin in their T2 seedlings. The latter ones consisted of nontransformed shoots that escaped the selection or of transformed shoots with low-expressed chimeric genes (data not shown); they were not used for mutation analysis.

Genetic mutation analysis of nontransformed and transgenic Arabidopsis lines

To determine the tissue culture-induced mutation frequency resulting from our leaf disc regeneration procedure, we screened 73 T2 populations of leaf disc-derived regenerates for recessive mutations as described in the Materials and methods and could identify one recessive mutation, classified as "thiamine-deficient" (Table 1). We concluded that the leaf disc regeneration procedure induced recessive mutations at a low frequency.

We chose 110 transgenic *Arabidopsis* lines transformed by either pGSATE1, pGSFR1161, or pGV2260::pGVp246 (Materials and methods) for mutational analysis. At first, for each of these lines, the T-DNA copy number was determined by looking at the ratio of sensitive to resistant T2 seedlings in selective germination, callus induction, or spray tests depending on the marker genes located on the T-DNA (Materials and methods). The number of T-DNA inserts determined by χ^2 analysis was verified in a DNA gel blot analysis for 29 of these lines. From these analyses, we concluded that each transformed line contains on average 1 or 2 T-DNA inserts, that each copy behaves as an independently segregating locus, and that the number of genetically determined T-DNA loci corresponds to the ones physically present. The overall number of T-DNA inserts within these lines was estimated to be about 150.

Following the described screening procedure, we found six recessive mutations within these 110 lines

Table 1. Genetic mutation analysis of nontransformed and transgenic *Arabidopsis* lines

Type of T1 plants	Mutations in total of T2	Mutant phenotype	Isolation number	Number T-DNAs[i]	Cosegregation T-DNA/mutation
Regenerants	1/40	Thiamine-deficient[b]	RUG-AR30	–	–
"pGV2260" regenerants[a]	0/33	–			
Transformants	6/110	Pointed first leaves (Pfl)[c]	RUG-AT50	4	Yes
		Miniature[d]	RUG-AT12	2	N.D.
		Gibberellin-sensitive dwarf[e]	RUG-AT11	1	No
		Gibberellin-insensitive dwarf[f]	RUG-AT64	1	No
		Auxotroph[g]	RUG-AT7	1	No
		Auxotroph[h]	RUG-AT98	2	No

[a] Leaf discs treated with nonvirulent pGV2260
[b] Rescued on minimal GM with 1 mg/l thiamine
[c] Differed mainly from wild type in the shape of the first two rosette leaves under normal growth conditions
[d] One-tenth the size of wild type
[e] Sensitive to 10 μM Ga4+7 for germination and elongation
[f] Normal germination, not sensitive to Ga4+7
[g, h] Developed few small yellowish leaves, lethal, restored partially to wild type on enriched GM
[i] Determined by DNA gelblot analysis–N.D., Not determined

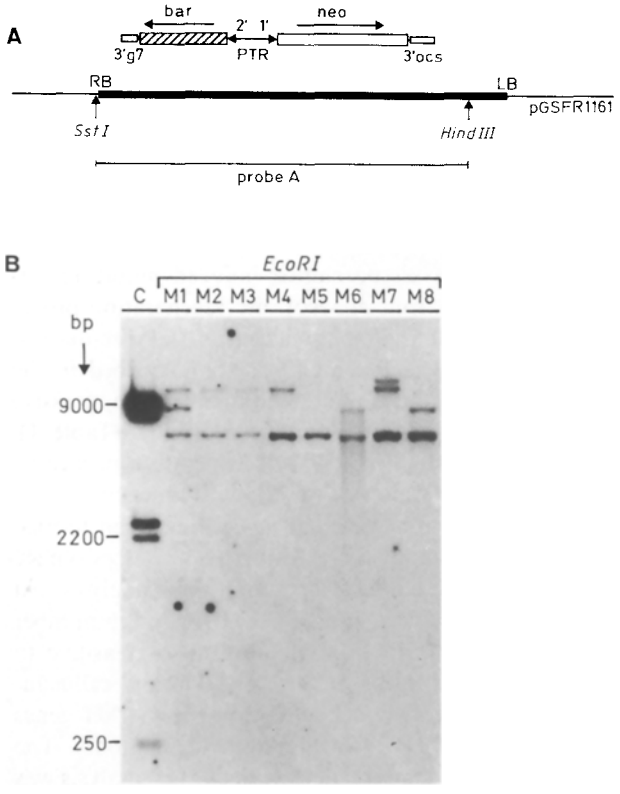

Fig. 1. A Schematic representation of the T-DNA region of pGSFR1161. The T-DNA flanked by the right (RB) and left (LB) border sequences is represented as a *black box*. Probe A is a 4-kb SstI-HindIII T-DNA fragment. The *bar* and *neo* chimeric genes are indicated. **B** Southern analysis on RUG-AT50-derived Pfl mutant individuals M1–M8. The molecular weight markers in lane C with homology to probe A are at a concentration of 10^{-3} µg. The hybridizing fragments indicate independently integrated T-DNA inserts in the plant genomes, since EcoRI does not cut within the T-DNA. One T-DNA insert is common to all mutant DNAs and is indicated. Hybridization conditions were as described in Materials and methods

(Table 1); they were all transformed by pGSFR1161. DNA gel blot analysis was used to look for co-segregation between a mutant gene locus and a particular T-DNA insert (Materials and methods). Among the dwarf and auxotrophic mutants, T2 individuals were found that did not contain a T-DNA insert (data not shown), an indication that these mutations arose from the tissue culture and/or infection procedure (Table 1). Not enough miniature mutant populations were tested to conclude co-segregation in this case. The 18 "pointed first leaves" mutants analyzed contained a common T-DNA insert amongst other segregating ones as illustrated in Fig. 1. These data indicated that the presence of a specific T-DNA insert was correlated with the *pfl* mutation. To determine whether the *pfl* mutation is T-DNA induced, an extensive genetic linkage analysis was carried out.

Tight genetic linkage between the pfl locus and T-DNA

One of the Pfl mutants, RUG-AT50/M5, was used as the male in a cross to the C24 wild type (Fig. 1 B). Chi-square analysis on the number of wild-type and mutant F2 seedlings (1,310 and 404, respectively) confirmed that the *pfl* mutation is nuclear recessive (χ^2 (3:1) = 1.87; $P > 0.1$).

To test the separability between the *pfl* locus and the kanamycin resistance marker on the T-DNA, F2 and F3 populations of the above-mentioned mutant cross to wild type were analyzed for recombinational events between *pfl* and *neo*, visible as PflKm^s plants. All 404 Pfl mutant F2 seedlings examined were Km^R in a selective callus-induction assay (Table 2). F3 progenies were harvested from 120 F2 mutant Pfl plants, and 100% of them consisted of Km^R seedings. From our F3 data and using a computer program from M. Koornneef (Koornneef and

Table 2. Measurement of genetic recombination frequency between the *pfl* locus and the Km^R marker on the linked T-DNA

Cross[a]	Scored[b] phenotypes	Number F2 seedlings	F3 populations with recombinants
C24 × RUG-AT50/M5	WT Km^S	434	–
	WT Km^R	939	–
	Pfl Km^R	404	0/120
	Pfl Km^S	0	–

[a] C24 is wild type (+ +/+ +) and RUG-AT50/M5 is a Pfl mutant containing the common T-DNA as unique insert, its genotype is *pfl neo/pfl neo* (Fig. 1; Table 1)
[b] WT, Wild-type first rosette leaves; Pfl, pointed first rosette leaves; Km^S, kanamycin sensitive; Km^R, kanamycin resistant

Table 3. Genetic mapping of the Km^R marker on the T-DNA linked to the *pfl* mutation

Cross[a]	Scored phenotypes[b]	Genetic class	Number F2 seedlings
W100 × RUG-AT/50/M5	WT Km^S	Recombinant	94
	WT Km^R	Parental	395
	An Km^S	Parental	140
	An Km^R	Recombinant	47

[a] W100, marker line, see Materials and methods; RUG-AT50/M5, see Table 2
[b] WT, Wild-type rosette leaves, wild-type siliques; An, narrow, dark green, angustifolia rosette leaves, crinkled siliques; Km^S, Km^R, see Table 2. The genetic distance was calculated with a certation coefficient of 0.8 for both the Km^R marker and the *an* locus

Stam 1987) that is based on the maximum likelihood procedure (Allard 1956) we calculated the genetic distance between the *pfl* locus and the *neo* gene on the T-DNA to be at maximum 0.4±0.4 cMorgans.

The T-DNA linked to pfl is located on chromosome 1

The homozygous mutant plant RUG-AT50/M5 was crossed with the marker line W100 (Materials and methods). We tested 676 F2 seedlings for kanamycin resistance (dominant) by the callus induction assay and determined the mutant phenotypes (recessive) of every seedling. The majority of the angustifolia (An) mutant plants were Km^s, namely 140 An/Km^s and 47 An/Km^R, indicating that T-DNA is linked to the *an* locus occupying position 0 on chromosome 1 (Table 3). The genetic distance between the *neo* gene on the T-DNA and *an* was calculated to be 19.0±1.83 cMorgan, as determined by the maximum likelihood procedure. Since our *pfl* mutation is closely linked to this mapped T-DNA, we concluded that the *pfl* locus maps onto chromosome 1 at or

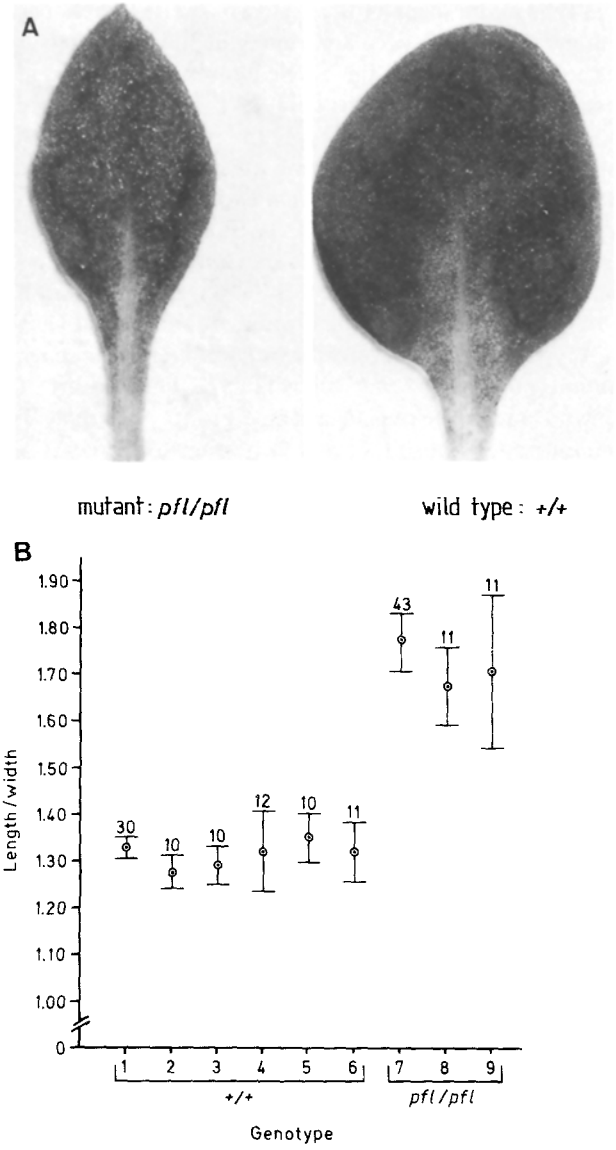

Fig. 2. A Comparison of first seedling leaves of wild type (+/+) and mutant (*pfl/pfl*) plants of 3-week-old sterile seedlings. **B** Graphical representation of means of leaf shape length/width values. Mean ($\bar{x}$) of length/width ratio is represented by *circled points*. *Error bars* indicate standard deviation σ to mean at 95% confidence. Sample size is given at each mean. Population 1, wild-type C24; populations 2–6, wild-type T2 populations derived from transformant RUG-AT50; populations 7–9, Pfl mutant T2 populations derived from RUG-AT50, named M5, M101, and M2, respectively

around position 19.0 cMorgan. It was not possible to map the *pfl* locus itself using this F2 population since the *an* locus to which *pfl* turned out to be linked also affects leaf morphology.

The "pointed first leaves" mutant phenotype

The "pointed first leaves" mutant (Pfl) originating from the transformant RUG-AT50 differs clearly from the

wild type in the shape of the first two rosette leaves. The mutant leaves are lanceolated, elongated, and pointed at the top in contrast to the cotyledon-resembling, round first leaves of the wild type (Fig. 2A). To describe more precisely the mutant phenotype, the mean length/width ratios with a standard deviation at the 95% confidence limits were calculated for the first two leaves in the wild-type and mutant populations. These are schematically represented in Fig. 2B. With the statistical sum of squares simultaneous test procedure (SS-STP) (Sokal and Rohlf 1981) on arc sin transformed ratios, we found that each wild type population is significantly different from each mutant population, that all wild type populations (1 2, ... , and 6) belong to a single group, and that all mutant populations (7, 8, and 9) belong to another distinct group. These data show that we can define populations that are mutant or wild type on a mathematical basis using length/width ratios of the leaf blade. Under normal growth conditions, mutant plants grow somewhat more slowly and are slightly retarded in flowering time with respect to the wild type. However, preliminary experiments indicate that different light intensities have a more dramatic effect on the mutant than on the wild type.

Discussion

We have studied the potential of the *Agrobacterium tumefaciens* T-DNA, which is naturally designed to function as a gene delivery system from the bacterium to the plant cell genome, to create recessive mutations in the small *Arabidopsis thaliana* genome under tissue culture conditions. *Arabidopsis thaliana* C24 was used throughout this study because of its high regenerative capacity in tissue culture (Valvekens et al. 1988; our own observations). Using leaf disc explants as starting material, we developed a rapid and efficient shoot regeneration procedure under a mild hormone regime, which has been shown to be important for achieving low somaclonal mutation frequencies (Scowcroft 1985). The presence of only one recessive mutation among 73 T2 populations derived from regenerated lines confirmed that optimal tissue culture conditions were used and that the procedure is suited for mutagenesis studies.

A transformation step with *A. tumefaciens* was incorporated into this regeneration procedure, and the efficient and rapid selection of transformed, fertile shoots either on kanamycin- or hygromycin-containing media was obtained. The transformation procedure and the co-integrative type of T-DNA vectors used caused a low-copy number of T-DNA insertion that facilitated further genetic mutation analysis. Using a standardized screening procedure (Materials and methods), we scored six recessive mutants in 110 T2 populations derived from transgenic lines. Southern analysis on several mutant plants of each of these six lines showed that only *pfl*, a mutation that mainly causes alterations in the morphology of the first rosette leaves under normal growth conditions, was co-segregating with a specific T-DNA insert. Four mutations segregated independently from their T-DNA insert(s), and one mutation that conferred a miniature size to the plants needs further investigation. If the higher somaclonal mutation rate found in transgenic lines is significant in terms of overall numbers estimated, the extra selection pressure in the media or the *Agrobacterium* infection per se could be responsible for it. This could lead to a higher frequency of mistakes in the gene repair mechanisms of the dividing plant cells in tissue culture, which is thought to be involved in the origin of some somaclonally induced mutations (Lee and Phillips 1988).

The analysis of F2 seedlings from a cross between a wild type and a Pfl mutant containing the co-segregating T-DNA copy as a unique insert confirmed that the *pfl* mutant locus was inherited as a nuclear recessive. Within the F2 and F3 populations derived from this cross, no recombination events between the *pfl* locus and the kanamycin resistance marker on the T-DNA were found to be scored as Km^s, "pointed first leaves" seedlings. These analyses showed a tight genetic linkage between the *pfl* locus and the T-DNA insertion, and the distance being at maximum 0.4 ± 0.4 cMorgan or approximately 120 kb indicates that the mutation is T-DNA induced. Further evidence comes from molecular analysis in which we found that the T-DNA-flanking sequences hybridize to a unique plant DNA sequence that corresponds to a transcript (unpublished results). Definite proof will come from further molecular analyses of the T-DNA target region and subsequent complementation analysis.

Under our transformation conditions we obtained one T-DNA insert that is closely linked to a detectable mutant gene locus, *pfl*, from a total of 150 T-DNA inserts represented in 110 transgenic lines. These data point towards a T-DNA mutation induction frequency between 0.5%–1% and indicate that *Agrobacterium*-mediated T-DNA transfer has the potential for the mutagenization of the *Arabidopsis* genome under tissue culture conditions. A similar T-DNA mutagenesis frequency was obtained by Koncz et al. (1990) using tissue culture. However, a significantly higher frequency of about 20% was obtained by Feldmann et al. (1989) using a seed transformation procedure that, so far, has proven difficult to repeat in other laboratories. The difference in T-DNA mutagenesis frequency found between a tissue culture and a non-tissue culture approach might be an indication that other or more target sites are exposed to T-DNA integration depending on the activation state of the cell genome and related to the transformation conditions used.

In the course of this work we established several genetic assays to test the activity of selectable marker genes in our transgenic lines and to facilitate the genetic linkage analysis of these markers to mutations. The results show that the *neo, hpt,* and *bar* chimeric marker genes and T-DNA gene 2 are expressed in a similar way in both *Arabidopsis* and tobacco. These findings imply that these genes can serve as genetic markers for introducing and following heterologous plant transposable elements in *Arabidopsis,* which are actually being studied as potential insertional mutagens in several laboratories. At present, no transposon-tagged mutants have been isolated in *Arabidopsis* (Schweizer et al. 1990).

The *pfl* mutation we isolated is located on chromosome 1 around position 19. It causes pointed first leaves and other minor visible changes in the plant development as explained in the Results sections. However, dramatic phenotypic effects can be obtained when using different light intensities. To determine which cellular processes are affected by the *pfl* mutation physiological and biochemical tests will be carried out. The study and manipulation of the gene determining the *pfl* locus that will be obtained from cloning the T-DNA target region will provide us with crucial information on the function of the gene.

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