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Update section

Mini review

T-DNA insertional mutagenesis in *Arabidopsis*

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Introduction

T-DNA insertion mutagenesis is one of those recently developed genetic techniques which can be expected to have a major impact in plant molecular biology [17, 50, 87]. Unlike conventional reviews, this brief outlook intends to give a practical insight into current problems and future applications of T-DNA tagging by underlining the potential of the genetic approach in studies of plant development.

Highlights of the past

T-DNA, a mobile element of *Agrobacterium* Ti and Ri plasmids, has become a universal tool for plant molecular biology. The term 'transferred DNA' (T-DNA) has much evolved in the past decade. Now, it refers to any DNA segment flanked by specific 25 bp direct repeats which can be transferred from *Agrobacterium* into plants by the help of Ti or Ri plasmid-encoded virulence (*vir*) gene functions provided in either *cis* or *trans*. Exploitation of the T-DNA as a plant transformation vector is based on the fact that it is stably integrated into the plant nuclear genome, hence its insertion into genes may cause mutations [35, 92]. To confirm the latter assumption, a promoterless kanamycin resistance gene was linked to the right-border repeat of the T-DNA and transformed into *Nicotiana tabacum* and haploid *N. plumbaginifolia* plants [2, 45, 79]. Selection for kanamycin resistance in both species resulted in transcriptional activation of the promoterless reporter gene exhibiting specific pattern of expression in diverse tissues of independent transformants. Plants transformed similarly by a reporter gene lacking the ATG initiation codon synthesized diverse kanamycin phosphotransferase fusion proteins. These data indicated that T-DNA integration can occur in protein coding regions of the nuclear genome yielding active transcriptional or translational plant gene-reporter gene fusions. The appearance of some morphologically altered *N. plumbaginifolia* plants, obtained after regeneration of spontaneous diploid shoots from haploid transgenic calli, also suggested that T-DNA insertions may inactivate or alter the expression of plant genes, the mutation of which results in 'loss of function' phenotypes [2, 45].

A generalized conclusion based on these early studies was that T-DNA tagging may become a suitable complement of classical transposon mutagenesis approaches in all plants for which

Agrobacterium-mediated transformation, tissue culture and regeneration methods are established. Since *Agrobacterium* has an extremely wide host range, applications of the T-DNA-tagging technique appeared to be of general importance. It was also foreseen that when combined with a gene fusion approach, the number of potential applications would be virtually unlimited.

From hypothesis to facts: successes, failures and corrections

Isolation of T-DNA insertions in actively transcribed chromosomal loci using a selection for the expression of *in situ* reporter gene fusions was a very attractive idea. By selection for the activation of promoterless kanamycin or hygromycin resistance genes in diverse explants (e.g. calli, roots or shoots exposed to various combinations of plant hormones) a large number of transgenic *Nicotiana* plants were regenerated. These plants expressed well-defined organ-specific gene fusions and displayed a normal Mendelian inheritance of selectable markers. Molecular analysis of over 200 transformants revealed, however, that the selection method resulted in a high copy number of T-DNA insertions. Selective probing of genomic DNAs with the promoterless marker gene from the right T-DNA end indicated that on average 5 to 20 T-DNA copies were inserted, whereas only few inserts hybridized with the left arm of the T-DNA. In contrast, control plants transformed by promoter-driven reporter genes (such as kanamycin or hygromycin resistance genes expressed by the promoters of the nopaline synthase or the mannopine synthase genes) carried intact T-DNAs at an average copy number of 1.5. This observation clearly indicated that a direct selection for T-DNA integration into actively transcribed plant genes leads to amplification of promoterless marker genes and integration of truncated, aberrant T-DNAs [46, and unpublished results]. Similar results obtained with a promoterless reporter gene placed into the middle of a T-DNA insert [32] suggested that there is a mechanism in plants by which foreign DNA is suitably processed and integrated into actively transcribed chromosomal domains. Although this mechanism hindered simple identification of mutagenic T-DNA inserts, it may well be exploited for experiments aiming for a targeted mutagenesis of specific loci, provided that a suitable selection can be found to distinguish between random integration and homologous recombination [5, 29, 54, 82].

A second lesson derived from these gene tagging attempts concerned the features of host plant species. *Nicotiana tabacum* has a complex genome, because this allotetraploid species carries full chromosomal complements of *N. tomentosiformis* and *N. sylvestris* ancestors. Therefore, apart for the successful isolation of gene fusions and the occurrence of rare dominant mutations, a mutagenesis approach in this plant may be of little benefit. One may reach similar conclusions when application of antisense or ribozyme constructs is considered, since analogous genes from *N. sylvestris* and *N. tomentosiformis* genomes are considerably divergent. Yet, a number of altered phenotypes resulted from transformation experiments, such as sterility, narrow leaf, dwarfism or variegation of petal colour and morphology. Only few of these traits would breed true in subsequent generations. Although such mutants are often classified as somaclonal variants, one can easily recognize polyploidy or aneuploidy associated with some of these phenotypes [27, 71].

Haploid *N. plumbaginifolia*, in contrast, was considered to be an ideal target since recessive mutations causing altered phenotypes can immediately be scored in the population of primary transformants [2]. This may be a disadvantage when genes regulating organ development or cell differentiation are studied, because homozygous mutants impaired in development cannot be regenerated to fertile plants. In a typical experiment (C. Koncz, unpublished) using direct selection for gene fusions after cocultivation of haploid protoplast with *Agrobacterium*, 300 tetraploid, 540 diploid and 10 haploid transformants were obtained. Together with cytological observations, this data showed that during cocultivation nuclear divisions without subsequent cell division do occur. As a consequence of nuclear endoreduplication,

over 30% of active gene fusions showed segregation after selfing of the primary transformants, indicating that T-DNA integration occurred after duplication of chromosomes. Among the diploid transformants 82 putative mutants were identified which displayed dwarfism, male or female sterility, albinism or altered morphology of diverse organs. Following outcrosses with diploid wild type only one albino carpel, a female-sterile and a dwarf (gibberellin synthesis) mutant were found to breed true to the F2 generation. Unexpectedly, cross-pollination of the wild type with the female-sterile mutant yielded both wild-type and female-sterile F1 hybrids indicating that androgenesis may occur in haploid-derived *N. plumbaginifolia*. Genetic analysis of these mutants was very time-consuming because *N. plumbaginifolia* seeds have an extremely long dormancy period which can only be broken by treatment with high concentrations of gibberellic acid. Nonetheless, after repeated outcrosses a close linkage could be established between the albino carpel mutation and a T-DNA insert which has been used to clone the corresponding gene. Isolation of genes from *N. plumbaginifolia* requires considerable experience. Representative genomic libraries can only be constructed in particular *Escherichia coli* hosts (e.g. strains carrying *mcrA, mcrB, hsdRMS, recBCJ and sbcC* mutations), because of a high level of methylation and abundance of GC-rich repeats in the nuclear DNA of this species. Thus, in spite of the advantage of high-frequency leaf-disc and protoplast transformation systems, *Nicotiana* species appeared to be difficult targets for these kind of gene tagging experiments.

Arabidopsis: *the ideal model*

Short life cycle, excellent genetics, small genome size, low content of repeated DNA, low level of methylation, efficient chemical and radiation mutagenesis are often referred as reasons why *Arabidopsis* was chosen as a model for plant molecular biology [15, 60, 61, 70]. Rediscovery of ideal genetic features of *Arabidopsis* coincided with efforts aiming to further develop gene tagging systems. Two independent, but interacting approaches, transposon and T-DNA tagging, were initiated. For both approaches it was essential to establish an *Agrobacterium*-mediated transformation system because, apart from a family of defective retroposons, no active transposable element could be identified in *Arabidopsis* [26, 67, 86]. Reports on successful tissue culture and regeneration were available and used in cocultivation of leaf, stem and root explants with *Agrobacterium* by diverse laboratories [for review see 50]. A common scheme for these tissue culture methods involves preconditioning of explants with high auxin to low cytokinin concentration ratios (e.g. 0.5 mg/l 2,4-D and 0.2 mg/l kinetin or 2.0 mg/l indole-3-acetic acid, 0.5 mg/l 2,4-D and 0.2 mg/l kinetin) followed by induction of shoot formation and embryogenesis in media containing high cytokinin to low auxin hormone combinations (e.g. 5.0 mg/l isopentenyl adenine and 0.1 mg/l naphtaleneacetic acid or 2,4 D). Although the recipes are quite similar, experience with tissue culture transformation is strikingly different in diverse laboratories, as discussed below. Using *Agrobacterium* infection of seeds and regenerating meristems, alternative *in planta* transformation techniques were developed, which have been reviewed recently in detail [17, 18].

Because of the lack of endogeneous transposable elements, maize transposon constructs, shown to be active in tobacco, tomato or rice [4, 13, 30, 62, 65], were introduced by T-DNA transformation into *Arabidopsis* but yielded surprisingly low transposition frequencies. A new design of one or two element *Ac/Ds* and *En/Spm* chimaeric transposons became therefore necessary which can efficiently excise from the T-DNA and reinsert into diverse genomic loci, if possible in the male or female gametophytes [11, 28, 42, 75, 78]. Along with T-DNA tagging, this technical correction made transposon tagging in plants a method of wide practicability.

Hunting for mutants

To demonstrate that gene mutations can efficiently be induced by T-DNA tagging in *Arabidopsis*, offspring of transgenic plants obtained by

either seed or tissue culture transformation have been screened for mutations influencing visible traits. Feldmann *et al.* [19] reported first that in a population of 136 seed transformants 36 mutations causing dwarf, agamous and glabrous phenotypes appeared, and demonstrated co-segregation of a dwarf mutation with the kanamycin resistance marker of the T-DNA of cointegrate Ti plasmid vector pGV3850:1003. By tissue culture transformation using improved gene fusion vectors several thousand transgenic *Arabidopsis* lines were generated simultaneously [72]. To avoid direct selection for expression of gene fusions, the new tagging vectors carried a functional hygromycin resistance gene as selectable marker, and a promoterless kanamycin phosphotransferase gene linked to the right T-DNA border, as a reporter. To facilitate the recovery of T-DNA inserts by plasmid rescue [44], a ColE1 replicon and a bacterial selectable marker were also inserted in the T-DNA tagging vectors (Fig. 1). Following selection for hygromycin resistant transformants, the frequency of T-DNA inserts inducing active reporter gene fusions was determined by monitoring the synthesis of kanamycin phosphotransferase enzyme in diverse tissues of transgenic plants, as well as by germination of M2 progenies on media containing kanamycin. In a population of 344 plants carrying a transcriptional gene fusion vector (pPCV621) 30.9 % of transformants expressed reporter gene fusions in diverse vegetative organs and 16.8 % of seedlings displayed selectable kanamycin resistance. From 134 transformants tagged by a translational gene fusion vector (pPCV6NFHyg) 32.4 % of plants expressed detectable kanamycin phosphotransferase enzyme activities and 20 % conferred selectable kanamycin resistance [46, and unpublished results].

Analogous experiments performed in tobacco and *N. plumbaginifolia* resulted in similar frequencies of T-DNA induced gene fusions. Segregation and DNA hybridization analyses indicated that the average copy number of T-DNA inserts in all three species ranged between 1 and 2. Since both size and complexity of *Arabidopsis* and *Nicotiana* genomes differ considerably, a comparable frequency of gene fusions indicated that T-DNA is frequently integrated into chromosomal loci

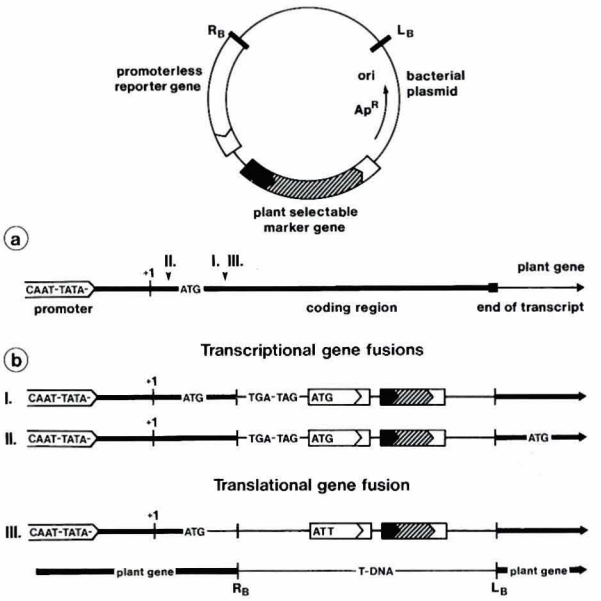

Fig. 1. Insertional mutagenesis with T-DNA gene fusion vectors. a. Schematic structure of gene fusion vectors carrying a promoterless reporter gene (e.g. *aph*(3′)II kanamycin phosphotransferase in vectors pPCV621 and 6NFHyg, 46, 87) linked to the right T-DNA border (R_B), a plant selectable marker (e.g. a nopaline synthase promoter-driven hygromycin resistance gene), and a bacterial plasmid replicon (Ap^R and *ori*) joined to the left T-DNA border (R_B). Underneath the vector a plant gene is depicted schematically with its promoter region (CAAT-TATA), transcription start (+1), translation start (ATG) and transcription termination sites. Target sites of putative T-DNA insertions are marked by arrows I, II and III. b. Principle of T-DNA gene fusion tagging. Line I shows a T-DNA insert in the coding region of a plant gene. In line II a T-DNA insert is located in the transcribed but untranslated leader of a plant gene. Insert I results in a dicistronic transcript because upstream of the ATG codon of the reporter gene stop codons are located in all reading frames. The first coding region of this dicistronic transcript encodes a truncated plant protein, while the second one encodes the reporter enzyme. Insert II yields a monocistronic transcript starting at +1 position of the plant gene and terminating at the polyadenylation site of the reporter gene. From both inserts I and II an intact reporter enzyme is synthesized. A T-DNA insert with a reporter gene that does not contain an ATG codon is shown in line III after integration into the coding region of a plant gene. T-DNA insert III results in an in-frame fusion between plant gene and reporter gene. This gene fusion encodes a fusion protein that consists of an N-terminal plant protein domain and a C-terminal reporter enzyme domain.

which are potentially transcribed. From 450 tissue culture-derived *Arabidopsis* transformants 137 segregated various mutations affecting size, morphology, colour, fertility, flowering time, germination, seedling viability and hairiness. From this population a yellow (chlorata) mutant was characterized in detail [48].

From T-DNA tags to genes

The genetic and molecular analysis of these T-DNA induced mutations documents both the simplicity and the efficiency of gene tagging approach. T-DNA tags provide dominant markers for genetic mapping, reporter genes for studies of transcriptional regulation, as well as molecular probes for gene isolation and complementation of mutations all in one. These advantages recently led to better understanding of the function of *Gl1* (glabrous), *AG* (agamous) and *CH42* (chlorata) genes in *Arabidopsis*.

The glabrous mutant, *gl1–43*, was identified as a seed transformant carrying two separate T-DNA-tagged loci. Genetic analysis indicated co-segregation of a hairless stem phenotype with one of these T-DNA inserts. Crosses with known hairless mutants revealed that the insertional mutation was allelic with, and dominant over, the *gl1* mutation located on chromosome 3 [57]. Physical mapping showed that the mutant carried at least 4 tandem T-DNA copies integrated at the same locus. A complicated structure of inserts prevented a simple plasmid rescue, therefore plant DNA fragments flanking the T-DNA tag were cloned from a λ library and used as probes for subsequent isolation of homologous wild type DNA. Nested deletions of wild-type genomic clones were transformed by a T-DNA vector into the *gl1* mutant which lacks trichomes on both stem and leaves. By assaying for complementation of the glabrous phenotype, the physical map position of gene *GL1* was determined [31]. Nucleotide sequence of gene *GL1* and its cDNA revealed a coding region for a Myb-like DNA-binding protein, which when used as a probe, detected a large family of homologous genes in the *Arabidopsis* genome. T-DNA integration in the mutant, *gl1–43*, occurred at 730 bp 3′-downstream of the Myb-coding region. The *GL1 myb* gene itself is not expressed in trichomes, but rather in leaf stipules. The intriguing questions why this T-DNA tag suppresses the activity of gene *GL1* in stem but not in leaf, and how *GL1* expression in stipules exerts its effect on trichome differentiation remain to be answered [63].

Characterization of agamous *ag2*, a second mutant from seed transformation, has opened the way to understanding flower differentiation. Isolation of the *agamous* gene (*AG*) followed a different strategy [91]. By crossing heterozygotes of T-DNA mutant *ag2* and a known EMS(ethylmethane sulphonate)-induced mutant, *ag1*, allelism was established. A junction fragment between T-DNA insert and plant DNA was isolated by plasmid rescue and used as probe: (a) for RFLP mapping which confirmed lack of recombination between the T-DNA insert and *ag1* mutation; (b) for isolation of a cosmid clone which complemented the *ag1* mutation; and (c) for isolation of a cDNA which failed to detect a complementary transcript in the T-DNA mutant, *ag2*. Characterization of wild type and mutant alleles showed that in the *ag2* mutant the T-DNA was integrated into the second, largest intron of gene *AG*, whereas in the EMS mutant *ag1* the acceptor site of the fourth intron was destroyed by a nucleotide exchange.

The *AG* gene is expressed exclusively in stamen and carpels and encodes a protein which is homologous to human SFR and yeast MCM1 transcription factors, as well as to the product of the homeotic *Antirrhinum* gene, *defA*. Both DEF A and AG proteins contain a common DNA-binding motif, the MADS box, which occurs in a large family of genes encoding transcription activators. Exploitation of MADS box homology led to discovery of further elements of homeotic gene families regulating flower development in *Antirrhinum, Arabidopsis*, and recently in tomato [37, 39, 55, 68, 76, 77]. T-DNA tags in *GL* and *AG* loci of *Arabidopsis* thus significantly contributed to recent progress in plant developmental biology.

A third mutation, chlorata (*cs*), derived from tissue culture transformation. Analysis of the *cs* mutation illustrates the exceptional precision of *Arabidopsis* genetics [48]. The mutation was assigned to chromosome 4 by trisomic crosses, then mapped by coupling and repulsion using known flanking markers. The resolution of genetic mapping was between 0.04 and 0.07 cM. In *Arabidopsis* this corresponds to about 6 to 11 kb, the size of the T-DNA insert which was thus found to be linked to the *cs* mutation. Map position of the *cs* mutation was identical with that of a known X-ray-induced chlorata mutation, *ch42*. Whereas the homozygote *cs* mutant is viable in soil, the *ch42* mutation causes homozygote lethality. Crosses between homozygote *cs* and heterozygote *ch42* lines confirmed allelism. A single T-DNA insert with flanking plant DNA was isolated from the *cs* mutant by plasmid rescue and used as a probe for cloning the wild-type gene and its cDNA, as well as the *ch42* allele. DNA sequence analysis revealed that in the *cs* mutant the T-DNA integrated into the C-terminal domain of the coding region resulting in the synthesis of a partly functional fusion protein. In the X-ray induced *ch42* mutant a deletion disrupted the coding region. Transformation of *cs* and *ch42* mutants with a wild-type genomic clone, as well as with a full-length cDNA driven by diverse promoters resulted in complementation of both chlorata mutants to green wild type.

The *CH42* gene is light-regulated and its product is transported into the chloroplast. Physiological analysis revealed that chloroplasts of the *ch42* mutant completely lack grana and PSII proteins, and are therefore sensitive to photooxidation. Interestingly, exposure to light and subsequent photooxidation arrests the expression of light regulated genes, including that of gene *CH42* itself. In both *cs* and *ch42* mutants protoporphyrin IX accumulates indicating the lack of functional protoporphyrin-Mg^{2+} chelatase, a key enzyme of chlorophyll biosynthesis (T. Falbel, personal communication). A failure to purify and assay this enzyme so far hindered basic studies on regulation of chlorophyll synthesis [90]. Gene probes and expression clones obtained from T-DNA tagging are now available to resolve this biochemical problem.

A wealth of mutants: some not linked to the T-DNA insert

The fascinatingly simple route from T-DNA tags to genes, and ultimately to solution of basic and applied biological problems, has greatly encouraged researchers to screen available T-DNA-tagged *Arabidopsis* collections. Laboratories from various fields of plant molecular biology have inspected 8000 seed transformants in the past two years and have identified an impressive collection of mutants displaying a wide spectrum of developmental and physiological alterations. Recessive mutations showing monogenic or digenic inheritance were scored in segregating populations representing on average 100 plants using either a visual screening for altered phenotypes in Petri dishes or in soil, or under defined selective conditions (e.g. for resistance or sensitivity to plant growth factors, cold, chlorate, etc.). Over 1000 putative mutants were identified. About 80% of these mutants showed a 3:1 or a 15:1 segregation of T-DNA-encoded kanamycin resistance marker and recessive mutant phenotypes. It was assumed that most mutations resulted from inactivation of genes by T-DNA insertions [17]. Others, screening smaller collections of tissue culture derived transformants, enjoyed less success. Van Lijsebettens *et al.* [85] found 7 mutants in a collection of 110 transgenic lines representing 150 T-DNA inserts. From these only one mutation, *pfl*, influencing leaf development showed cosegregation with a T-DNA insert. Although screening data from other laboratories were not reported, it became the general opinion that tissue culture transformation is an obstacle to tagging because the majority of derived mutants are somaclonal variants. As discussed above, a very similar argument was employed earlier when T-DNA tagging led to practical difficulties in *Nicotiana* species without resolving the problems. How can one explain the striking difference between mutation frequencies

reported in tissue culture and seed transformations? To answer this question, several facts ought to be considered.

Currently little is known about the fate of T-DNA during transformation. Nucleotide sequence comparison of T-DNA insert junctions and target sites indicated that T-DNA integration in the plant genome occurs by illegitimate recombination including double-stranded break and gap repair [23, 58, 59]. The VirD2 protein, which targets the T-DNA to the nucleus [33, 36], probably plays an important role in the recognition of nicks and breaks that serve as substrates (i.e. entry sites) for integration. Most integration events yield small target site deletions and involve limited pairing between T-DNA and target plant DNA sequences during repair DNA synthesis. It is not known whether all T-DNA copies attacking various sites in the nuclear genome are being stabilized by replication. It may well be that abortive integration events, in which the T-DNA (i.e. T-strand) is removed by repair, leave their 'footprints' in the nuclear DNA. Such hypothetical 'footprints' may be as small as single base-pair exchanges or deletions. Preliminary data indicate that in a portion of T-DNA tagged lines chromosome breakage and/or translocation can also occur as a consequence of double-stranded break repair. These events equally influence the frequency of mutations that occur independently of T-DNA insertions both in tissue culture and seed transformation.

As in *Nicotiana*, endoreduplication of chromosomes has also been observed in *Arabidopsis*, and is suspected to cause chromosomal aberrations, polyploidy or aneuploidy in tissue culture [21]. Seed transformation apparently avoids this problem probably because polyploidization does not occur in meristemic and reproductive cells. It is well known that most aneuploids are semisterile in *Arabidopsis*, and that tetraploids do not segregate mutations at 3:1 or 15:1 ratios. Plants with higher ploidy regenerate from tissue culture very inefficiently. Genetics, even a single outcross with wild type, can help to detect aneuploids or tetraploids, because in the first case semisteriles and morphological variants appear in F1, whereas in the latter case the resulting triploid cannot breed true [71].

It may be argued that tissue culture includes a variety of synthetic plant growth factors, some of which can cause mutations in *Arabidopsis*. 2,4-D (2,4-dichlorophenoxyacetic acid) has been observed to induce heritable phenotypic alterations in several species after regeneration from tissue cultures. High-frequency transformation and rare somaclonal variation achieved in tissue culture transformation implies that 2,4-D was used at low concentrations and only for a transient period during auxin conditioning of explants [50]. A number of other events (e.g. alteration of methylation pattern, somatic recombination, induction of silent transposons) may increase mutation rates in tissue culture. However, even taking all these events together one can hardly explain the 10-fold difference observed between frequencies of T-DNA-induced mutations in seed and tissue culture transformation experiments.

Although the main aim of our approach was the technical development of insertion mutagenesis and the analysis of tagged developmental mutations, recently a detailed characterization of 1340 tissue culture derived transgenic *Arabidopsis* lines was initiated by friendly collaboration of 9 laboratories. A screening scheme as that applied for seed transformants was followed [17]. A spectrum of mutant phenotypes and their frequencies in the collection are summarized in Table 1 and compared to data derived from screening of seed transformants. In spite of probable differences in the definition of certain phenotypes, the data show surprising similarity. When the frequencies of albinos (which can be classified safely) are compared to data from an EMS mutagenesis experiment [40], a good correlation is apparent. Nonetheless, it would be premature to conclude that T-DNA is as efficient a mutagen as EMS, although the present analysis of chromosomal location and target sites of insertions indicates that T-DNA integration has no locus or sequence preference [1, 9, 23, 58, 59, 89].

In our view, the present debate on the potential of diverse T-DNA tagging approaches is premature since it lacks supportive genetic data.

Table 1. Mutation spectrum of seed and tissue culture transformation experiments.

Altered trait*	Seed infection[1]		Tissue culture	
	number of plants	%	number of plants	%
I. **Size/Viability**				
Seedling lethal	240–400	3–5	8	0.59
Size variant	240–400	3–5	74	5.52
Embryo defective	200–280	2.5–3.5	74	5.52
Reduced fertility	80–160	1–2	3	0.22
Steriles	15	0.2	2	0.14
II. **Pigment**				
High anthocyanin			25	1.86
Fusca	8	0.1		
Albino (EMS: 1.18 %)[2]	*95*	*1.2*	*15*	*1.12*
Yellow-green	104	1.3	38	2.83
Dark-green	16	0.2	7	0.52
III. **Organ development**				
Flower	40–48	0.5–0.6	3	0.22
Root	8–24	0.1–0.4	37	2.76
Root hair	28	0.35		
Trichomes	20	0.25	11	0.82
Dwarfs	12	0.25	10	0.74
IV. **Physiological**				
Flowering time	40	0.5	6	0.44
Wax production	8	0.1		
High fluorescence	8	0.1		
Auxotrophy			2	0.15
Fatty acid synthesis			3	0.22
Other			18	1.34
Total	1200–2080	15–26	336	25.07
Population screened	8000		1340	

[1] Frequencies reported by Feldman [17] were converted to numerical values.
[2] Frequency of albino mutants detected by Jürgens *et al.* [40] in an EMS mutagenesis experiment.
* All data derived from primary screening of seed or tissue culture transformants are preliminary and may change as segregation and linkage analysis proceed.

Scores for altered phenotypes in M2 populations (termed T3 generation in seed transformation) summarized in Table 1 by no means indicate that the mutations are actually caused by T-DNA insertions, even if they displayed correct Mendelian segregation ratios of T-DNA markers and mutant phenotypes. Analysis of linkage between mutations and T-DNA inserts requires further careful inspection of a large number of M2 and M3 progenies and/or detection of cosegregation of these markers in F2 and F3 generations following outcrosses. Preliminary data from such analyses of both seed and tissue culture transformants indicate that possibly only 10 to 40 % of mutations are caused by T-DNA inserts [14; C. Koncz, unpublished; K. Feldmann, personal communication]. This is of course somewhat annoying, but when it is considered that current collections may provide over 800 T-DNA tagged loci, the expected input of tagging in *Arabidopsis* molecu-

lar genetics is still remarkable. 'Unlinked' mutations derived from tagging are also not wasted because they may be exploited for gene isolation by RFLP mapping and chromosome walking [38, 49].

Future trends: technology development and saturation mutagenesis

It is estimated that isolation of 100 000 T-DNA transformed lines will yield at least one tag in all average size genes in *Arabidopsis* at a probability of 95%. The success of a saturation mutagenesis approach requires further technical development. Doubts regarding the usefulness of such an approach are due to the belief that insertional mutagenesis by T-DNA can yield only 'null mutations', which by causing homozygote lethality may prevent the identification of particular genes. Examples of tags within the *ch42* and *gl1* loci clearly show the weakness of this argument by demonstrating that T-DNA inserts, as EMS, may result in leaky 'loss of function' mutations. Eventually, this problem can be resolved by application of novel techniques described below.

Toward high-frequency transformation: learning more about Agrobacterium-*plant interaction*

The unique observations that *Agrobacterium*-mediated infection of seeds and regenerating meristems can provide T-DNA transformed offspring underlined the importance of further studies of *Agrobacterium*-plant interaction. A detailed understanding of the molecular mechanism underlying *in planta* transformation is very important for optimization of these transformation methods. Recent results of *Agrobacterium* genetics provide suitable tools for immediate improvements [see for review 35, 50].

Successful seed transformation has been achieved so far only with cointegrate T-DNA vectors derived from Ti plasmid C58. It is apparent that properties of VirA proteins encoded by diverse Ti and Ri plasmids are considerably different, and dramatically influence both host range and transformation efficiency of *Agrobacterium* strains [83]. VirA is a membrane-bound chemical sensor, which is activated by plant phenolics, sugars and low pH. VirA-mediated phosphorylation of transcription factor VirG is required for activation of *virB, C, D, E* and *F* operons that encode proteins mediating the processing and transfer of the T-DNA. VirA of Ti plasmid C58 has a wider pH optimum and differs in its ligand specificity to various phenolics from VirA proteins of other Ti and Ri plasmids. VirA from C58 permits an efficient DNA transfer also to monocots [74], whereas VirA proteins of other *Agrobacterium* strains are severely inhibited by certain phenolics and phytoallexins produced by these plant species. Furthermore, the *virG* locus of pTiC58 seems to be constitutively expressed, while in other strains its activity is negatively controlled by the PhoB repressor of phosphate regulons. Recently, several VirA proteins were engineered, which are active at various pH conditions in the absence of inducing phenolics and sugars. Similarly, diverse chimeric *virG* genes were constructed, which permit constitutive or inducible expression of virulence genes in *Agrobacterium* [3, 7, 8, 66, 83].

Studies of T-DNA transfer during *Agrobacterium* infection of intact plants using an intron containing *uidA* reporter gene indicated that *Agrobacterium* can systemically infect most plant tissues and transfer its T-DNA to various cells [25, 84]. It is thus conceivable that infection may result in a transformed lineage of cells, if infected cells were able to divide. This also suggests that *in planta* transformation by *Agrobacterium* does not need artificial wounding to produce activating phenolics. The discovery of an *Agrobacterium* β-glucosidase which releases a *vir*-inducing phenol, coniferyl alcohol from its glucoside, coniferin, supports this hypothesis [6, 34, 64]. In summary, tools and ideas to study the mechanism and improve the efficiency of *in planta* T-DNA transfer are available.

Concominant development of tissue culture methods aim to decrease the labour involved in the regeneration of *Arabidopsis* transformants. Methods which do not require explant transfer

during subcultures and permit processing of large numbers of transformants are being developed. Co-cultivation of protoplasts with *Agrobacterium* followed by induction of embryogenesis is another major goal of current efforts.

Stability of T-DNA vectors is an important aspect of technology development since most popular vectors are based on plasmid RK2-derived minireplicons which lack partitioning functions. A locus regulating resolution, and thereby stability, of plasmid RK2 has been isolated, and plasmid derivatives maintained stably in the absence of any selection will soon be available for both *in planta* and tissue culture transformations [22, 73]. Some binary vectors used in combination with disarmed derivatives of Ti plasmid C58 are now also converted to cointegrate forms to facilitate the optimization of *in planta* transformation methods [87].

Gene fusions: activation in cis *and* trans

The gene fusion approach remains a 'powerhouse' providing versatile applications in tagging. As a side product of early insertion mutagenesis experiments in *Nicotiana* and *Arabidopsis*, gene fusions were exploited for the isolation of specific plant promoters expressed in root or root hairs [46]. Characterization of the first T-DNA tagged promoters revealed that dicistronic transcripts are translated in plants and indicated that plant promoters separated by various distances *in cis* may modify (ie. stimulate or silence) the activity of each other in a tissue/organ-specific fashion. It was soon realized that virtually any gene can be applied as reporter in combined gene fusion-tagging experiments, given the availability of a suitable assay system.

Exploitation of a promoterless *uidA* (β-glucuronidase) reporter gene linked to the T-DNA border thus provided a sensitive *in vitro* histological staining procedure for identification of gene fusions expressed in various tissues of transformed plants [20, 43]. By insertion of a minimal TATA box promoter between the T-DNA border and *uidA* reporter gene, vectors for detecting specific enhancers were employed with similar success [24, 81]. Application of fused bacterial luciferase genes, *luxA* and *luxB*, as reporters allowed the detection of temporal and spatial activity of gene fusions by monitoring an image of luciferase-mediated light emission in intact living plants [47, 53]. These applications further confirmed the observation that T-DNA frequently integrates into transcribed chromosomal loci and increased the number of T-DNA-tagged genes and promoters in *Arabidopsis*.

As the potential of the tagging approach was demonstrated, the need for applications in other plant species has increased. Thus, genetic dissection of important biological processes, such as wood production, nitrogen fixation or alkaloid production, by gene fusion tagging is now feasible due to the availability of high-frequency *Agrobacterium* transformation systems in various species (e.g. poplar, alfalfa or tobacco). How can genes involved in such specialized functions be identified by tagging in a diploid or tetraploid organism? A solution to this question, derived from the observation described above, that strong enhancers, such as those carried by the promoter of CaMV 35S RNA, can *cis*-activate the expresssion of other genes located within a short distance in the plant genome.

To activate gene expression by T-DNA tagging, multiple copies of -90 to -360 enhancer domains of CaMV 35S promoter were placed close to the right border of the T-DNA and tagging vectors were introduced into tobacco by protoplast cocultivation. It has been assumed that activation of genes involved in the synthesis or signal transduction pathway of auxin will result in rare transformants that are able to develop without exogenous supply of this growth factor. Indeed, several transformants were obtained from which the re-isolated T-DNA tags and flanking plant genes conferred auxin-independent growth when re-introduced into plants [88; R. Walden, personal communication]. In a similar fashion any promoter with characterized enhancer or silencer elements can theoretically be exploited for activation or silencing of genes in various organs or tissues by T-DNA tagging. Since the resulting

phenotypes are dominant, the application of such 'activator' or 'silencer' tagging strategies is not dependent on the ploidy level of mutagenized plants. In addition 'gain of function' mutants can be selected more conveniently than 'loss of function' mutants, and are often of greater importance to understand the physiology of plants. Finally, such 'gain of function' mutants are not likely to result from somaclonal variation.

It is impossible to cover all combinations of gene fusion and tagging techniques in a mini-review. Two further examples should, however, receive some attention because they offer novel contributions to studies of development and gene regulation in plants. To identify genes expressed only in particular cells or during defined stages of development, a 'suicide' gene fusion technique can be employed. Several genes, the expression of which cause immediate or conditional cell lethality are available, such as the RNase T1 gene of *Aspergillus*, the barnase gene from *Bacillus amylolyquefaciens*, the diphteria toxin A chain gene or a *Pseudomonas* gene encoding exotoxin A [10, 56, 69, 80]. Expression of suicide genes in particular cells may result in dominant 'missing pattern' mutations. Application of T-DNA genes, which encode the synthesis or modification of plant growth factors auxin and cytokinin, may lead, in contrary, to 'gain of pattern' mutations by affecting hormonal regulation of cell differentiation (i.e. formation of chimaeric organs) [12, 16, 41, 52, 92]. Combination of diverse promoters to express marker genes in a tissue-specific manner with either of these approaches opens the way to tagging experiments by which mutations in particular regulatory genes can be identified. When gene families of transcription factors, cell cycle regulatory proteins or putative hormone receptors are taken into account as potential effector or reporter genes in such gene fusion-tagging experiments, the scope of these molecular genetic techniques can be enlarged even further.

Conclusion

The value of a technique can only be judged by its input in new research, and the evaluation should rely on firm experimental data. In case of T-DNA tagging, although the technique is still in its infancy and the data are incomplete, the input in plant molecular biology, and in particular in *Arabidopsis* molecular genetics, is already remarkable. We hope that, by confrontation of known and unpublished data and by discussion of future trends, this timely outlook will encourage further development of this fascinating technology.

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