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The use of transgenic plants to understand transposition mechanisms and to develop transposon tagging strategies

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Key words: transposition mechanism, *Ac*, *Tam3*, *En/Spm*, *Mu*, two-element systems, transposon tagging, transgenic plants.

Abstract

This review compares the activity of the plant transposable elements *Ac*, *Tam3*, *En/Spm* and *Mu* in heterologous plant species and in their original host. Mutational analysis of the autonomous transposable elements and two-element systems have supplied data that revealed some fundamental properties of the transposition mechanism. Functional parts of *Ac* and *En/Spm* were detected by *in vitro* binding studies of purified transposase protein and have been tested for their importance in the function of these transposable elements in heterologous plant species. Experiments that have been carried out to regulate the activity of the *Ac* transposable element are in progress and preliminary results have been compiled. Perspectives for manipulated transposable elements in transposon tagging strategies within heterologous plant species are discussed.

The application of transposable elements in gene cloning: transposon tagging

Although the genome of most organisms is relatively stable mutations can occur by factors that induce genetic instability. Transposable elements are one of the factors that can cause DNA alterations and thereby create mutations. Plant transposable elements and the visible mutations they have caused, have been characterized most thoroughly in the plant species *Zea mays* (maize) [for reviews see 17, 52] and *Antirrhinum majus* (snapdragon) [10, for a review see 11]. When these transposable elements were cloned it became possible to isolate the genes that were mutated by the transposable elements. With the characterized transposable elements *Ac/Ds* (Acti-

vator / Dissociation) [15, 46], *En/Spm* (Enhancer / Suppressor-mutator) [47] and *Mu* (Mutator) [3, 17] from *Zea mays* and the *Tam3* transposable element from *Antirrhinum majus* [42] this strategy called transposon tagging proved to be feasible for plant gene cloning. Visual selection of a mutant was possible upon insertional mutagenesis with a transposable element and subsequently the gene fragment flanking the transposable element could be cloned using the transposable element as a probe [14, 62]. Most of the regulatory genes isolated using this strategy could not have been cloned from DNA libraries because no gene product was known. To extend this transposon tagging strategy to plant species of which endogenous transposable elements have not yet been cloned (i.e. crop plants like tomato, potato),

transformation techniques can be employed to introduce one of the characterized transposable elements. Because maize and snapdragon are not yet routinely accessible to genetic manipulation and because they contain many copies of related transposable elements in their genome, heterologous plants might be used as test tube for experiments regarding the mechanism and regulation of transposition. Introduction of a transposable element into such a virgin genetic background, in the original and mutagenized forms, will allow the determination of factors that are important in *trans* or in *cis* for the functioning of a mobile DNA element. In this review we consider the activity of the transposable elements *Ac*, *Tam3*, *En/Spm* and *Mu* in several plant species and compare their behaviour to that in the original host. The data that have been mounting on the mechanism of transposition and the regulation of its activity are compiled and examined for their applicability in transposon tagging strategies.

Activity of autonomous plant transposable elements in new genetic backgrounds

The major question that has to be addressed when a transposable element is introduced into a new genetic background concerns the activity of the element. Will the autonomous element perform the excision and integration process with the same accuracy in the new host? The introduction of the maize *Ac* element in tobacco showed that this transposable element behaves in a similar fashion in tobacco as it does in maize [1]. It excises from the flanking waxy sequence with high frequency, leaving behind the footprints characteristic of *Ac* [17] and reintegrates into the tobacco DNA thereby creating an 8 bp target site duplication. Although this molecular approach established that *Ac* is active in other plant species, the development of a genetic method to determine the excision of *Ac* or any other mobile DNA element greatly facilitated the analysis of transposition in other plant species. The phenotypic assay that was introduced by Baker *et al.* [2] relies on the restored expression of a marker gene after the

Fig 1. A phenotypic assay for the excision of a transposable element. Depending on the marker gene employed the excision of a transposable element can be selected for by the restoration of the activity of the NPTII gene (kanamycin resistance), HPT gene (hygromycin resistance), CAT gene (chloramphenicol resistance) or screened for in whole tissues or individual cells using visual reporter genes, GUS (β -glucuronidase) and rolC. The SPT gene (streptomycin resistance) can be used for both purposes when selection is performed on tobacco seedlings. The target site duplications that flank the transposable element and remain as a footprint after excision are diagrammed as open and hatched boxes.

excision of the transposable element (Fig. 1). An important function of this phenotypic assay is the estimation of the excision frequency (a measure for the activity of a transposable element) in somatic tissue but it can also be used to establish the germinal excision frequency by progeny analysis. In this way it has been established that *Ac* is active in the dicot and monocot plant species tested so far: *Nicotiana tabacum*, *Daucus carota*, *Lycopersicon esculentum*, *Solanum tuberosum*, *Arabidopsis thaliana*, *Glycine max* and *Oryza sativa* (see Table 1). The frequency of excision ranges from 1–80% depending on the assay employed, the plant species tested and the stage of plant development analysed. In somatic tissue *Ac* excises with an average frequency of 20–50% of the transformed calli or shoots. However, in some plant species it is very difficult to detect *Ac* transposition. With a phenotypic assay no excision of the *Ac* element could be detected in the tropical pasture legume *Stylosanthes* (J. Manners, CSIRO

Table 1. Activity of the autonomous transposable elements *Ac*, *En/Spm*, *Tam3* and *Mu1* in heterologous plants. The gene employed for the phenotypic assay is indicated while molecular analyses are denoted as mol. Results from analyses of primary transformants or regenerating calli have been considered as somatic transposable element activity (som.), while germinal excision frequencies have been determined by progeny analyses. The analysis of *Tam3* in tobacco was performed on a small progeny (*). Some assays to determine the excision frequency were based on the calculation of sectors of enzyme activity in a leaf (sectors). When the integration of the transposable element has been confirmed with molecular data this has been stated (+). ND: not determined.

	Assay		Excision freq.	Integration	Ref.
	Plant stage	Reporter			
Ac element					
<i>Nicotiana tabacum</i>					
	som.	mol.	44%	+	1
	som.	NPTII	27–70%	+	2
	som.	HPT	36%	+	27
	som.	GUS	(< 10%) sectors	ND	19
	som.	GUS	33%	Rommens, unpubl.	
	som.	rolC	ND	ND	54
	progeny	SPT	1–9%	+	36
	progeny	mol.	> 20%	+	57
<i>Daucus carota</i>					
	som.	mol.	28%	+	58
	som.	mol.	ND	+	59
<i>Arabidopsis thaliana</i>					
	som.	mol.	30%	+	58
	som.	NPTII	51%	+	50
	som.	mol.	0.2–0.5%	ND	50
<i>Solanum tuberosum</i>					
	som.	NPTII	50%	+	37
<i>Lycopersicon esculentum</i>					
	som.	mol.	80%	+	64
	progeny	mol.	ND	+	4
<i>Glycine max</i>					
	som.	GUS	45%	+	65
<i>Oryza sativa</i>					
	som.	HPT	35%	+	34
En/Spm element					
<i>Nicotiana tabacum</i>					
	som.	mol.	7%	+	48
	progeny	NPTII	5% sectors	ND	48
	som.	GUS	10% sectors	ND	44
	som.	HPT	ND	+	44
<i>Solanum tuberosum</i>					
	som.	mol.	ND	+	20
Tam3 element					
<i>Nicotiana tabacum</i>					
	som.	mol.	6–12%	+	43
	progeny	mol.	– *		43
	som.	HPT	43%	+	27
	som.	GUS	13%	ND	27
<i>Petunia hybrida</i>					
	som.	HPT	22%	+	27
		GUS	13%	ND	27
Mu1					
<i>Arabidopsis thaliana</i>					
<i>Nicotiana tabacum</i>					
<i>Lycopersicon esculentum</i>					
	som.	mol.	no activity		65

personal communication). Another plant species in which *Ac* is not very active is lettuce. Only when *Ac* cDNA is equipped with the T-DNA 2'-promoter excision can be measured with a phenotypic assay based on kanamycin resistance (C.H. Yang, R.W. Mitchelmore, U.C. Davis and J. Ellis, CSIRO, personal communication). These results indicate that the behaviour of *Ac* can differ in heterologous hosts.

It has been reported in maize that the germinal excision frequency can vary between 0.4% and 17% [5, 25]. In the analysis of the germinal excision in *Arabidopsis* a frequency of 0.2–0.5% has been determined [50]. However, a modified *Ac* element has a germinal excision frequency of 1–9% in tobacco [36], while in tomato a high frequency of germinal *Ac* excision has been reported after molecular analysis of a limited number of plants [4]. Just as stated for the somatic activity of *Ac*, it appears that differences between plant species influence the germinal excision frequency of *Ac*.

Molecular analyses demonstrate that in all plant species the maize *Ac* element reintegrates following the excision. Parameters characteristic of the transposition process, excision (frequency), re-integration and footprints, have been determined in heterologous hosts and the results allow the conclusion that the autonomous *Ac* element behaves similarly in heterologous plants compared to maize. A striking difference between the transposition of *Ac* in maize and tobacco concerns the *Ac*-dosage effect. In maize the increase of active *Ac* elements results in a decrease of transposition, while in tobacco more copies of *Ac* cause more transposition [30, 36].

A special feature of the phenotypic assays employing a cell autonomous marker gene [36, 54] is the fact that the timing of *Ac* excision can be defined, while even plant cell lineages can be followed [19] when marker gene re-activation takes place in specific cells. Other phenomena caused by *Ac* or *Ds* transposition are deletions and inversion occurring in the maize DNA [49]. It is to be expected that such observations will also be reported for heterologous plants that harbour copies of *Ac* or *Ds* elements.

Because the maize *Ac* element and the *Antirrhinum majus Tam3* element are regarded as related elements (due to their terminal inverted repeat length, the 8 bp target site duplication that is formed upon integration into the host DNA and sequence homology in the putative transposase coding region [53]) it was anticipated that *Tam3* would also be highly active in heterologous plants. Although the initial excision frequency of *Tam3*, after the introduction with *Agrobacterium* into tobacco, ranges from 10–40% [27, 43], further analyses suggested that *Tam3* subsequently can be inactivated efficiently by methylation. Still, the transposed *Tam3* elements did produce a target site duplication upon re-integration into the tobacco DNA [43].

The maize *En/Spm* element, a member of another family of structurally related transposable elements (*Tam1*, *Tgm1*, *En/Spm* [11, 47, 61]) has been introduced into tobacco [44, 48] and potato [20]. It excises in somatic tissues with a frequency of 5–10%, thereby exhibiting a lower activity than the maize *Ac* element. A phenotypic assay based on the restoration of the GUS gene was applied successfully to determine the excision frequency of the *Spm* element [44]. However, chimaeric HPT and NPTII genes were not completely inactivated by the insertion of the *En/Spm* element, making this phenotypic assay unsuitable for the selection of excision events [48]. Still, the integrity of the excision and integration process of *En/Spm* was established by sequencing the empty donor sites and the fragments flanking the new integrations in the tobacco DNA. The *En* element continues to transpose in the progeny of transformed tobacco plants [48].

The transposable element *Mul* has been a useful tool in transposon tagging in maize [14]. The small size of the cloned *Mul* fragment and the absence of an open reading frame large enough to code for a 'transposase' enzyme suggested that this copy is a non-autonomous element [17]. Transfer of *Mul* to tobacco, tomato and *Arabidopsis* [65] demonstrated that this element cannot transpose in these heterologous hosts. Either *Mul* is a non-autonomous element or it is dependent on specific maize enzymes absent in the new hosts.

Controlled transposon tagging with two-element systems

For two reasons the development of two-element systems is important: firstly, for mutagenesis studies of factors that act in *cis* or in *trans* during the transposition process and secondly, in transposon tagging strategies. A two-element system separates the enzymatic function(s) ('transposase') from the target sequences of the mobile DNA element. The non-autonomous element cannot transpose by itself but can be trans-activated by a transposase-producing element. The *cis*- and *trans*-acting functions within a transposable element can thus be dissected by mutational analyses.

For the maize transposable elements *Ac* and *En/Spm* natural non-autonomous elements have been characterized. These elements *Ds* and *I/dSpm* [15, 17, 23] are in most cases internally deleted forms of the autonomous transposable element (an exception is *Ds1* that only has homology to the inverted repeats of *Ac* and no homology to any other *Ac* sequences [55]) and are only active in the presence of an activator. These naturally occurring two-element systems were reconstituted by sequential transformation or by crosses of transgenic plants harbouring either of the two elements (Table 2). Transformation of *Ds*-containing tobacco plants with an *Ac*-containing vector resulted in activation of the non-autonomous *Ds* element with a frequency approximately 50% of that of the autonomous *Ac* element [12]. When *Ac* and *Ds* are combined by crosses of transgenic plants excision of the *Ds* element can be detected in the F1 progeny. In 24–50% of the F1 progeny of transgenic tobacco plants excision of an artificially constructed *Ds* (*Ac*Δ) element has been registered [29]. The *Ds* element integrated into the tobacco genome after excision from the NPTII marker gene. The presence of an *Ac* element in a tomato plant also harbouring *Ds1* resulted in almost all cases in *trans*-activation of *Ds1* [40]. The efficiency of *trans*-activation of the small (0.4 kb) *Ds1* element is significantly higher than the other *Ac*Δ elements that have a size ranging from 3.4 to 7.8 kb. The

Ac/Ds system can be reconstituted in a heterologous plant and can therefore be used to examine *cis*- and *trans*-acting functions of *Ac*.

Similar experiments to establish *trans*-activation of a *dSpm* element by an active *Spm* element have been performed in transgenic tobacco plants [44]. In this case the *trans*-activation of a 2.8 kb *dSpm* element from a GUS reporter gene was reported to be a more efficient process than the transposition of an autonomous *Spm* element. No data were presented on the integration of the *dSpm* element into the tobacco genome. Although a 2.2 kb *I* element was unable to inactivate the NPTII marker gene [48], molecular analysis showed that *trans*-activation by an *En* element was possible in tobacco. Transposition of the *I* element resulted in the expected footprint and upon integration in the tobacco DNA a target site duplication of 3 bp characteristic of *En/Spm* was created. As the two-element systems for *En/I* and *Spm/dSpm* appear to be active in heterologous plant species, studies on the functional domains of this transposon can be initiated.

Recently a two-element system for *Tam3* has been established in transgenic tobacco plants [28]. Since no naturally occurring two-element system has been described for *Tam3*, an artificial system has been constructed consisting of an immobilized *Tam3* element as the activator and a non-autonomous element in which a 1.3 kb internal fragment of *Tam3* has been replaced by a bacterial plasmid. The *trans*-activation frequency was estimated to be less than 5%, while re-integration of the non-autonomous element could only be detected in 16% of the plants. These data are consistent with the low activity of autonomous *Tam3* elements in heterologous plants, but sequence analysis of d*Tam3* excision sites suggests that the inefficient re-integration may be caused by imprecise excision. In the cases where parts of the ends of the d*Tam3* element remained in the marker gene or small deletions had occurred no re-integration of d*Tam3* could be detected. Conceivably, *Tam3* cannot perform the transposition process accurately without factors that are supplied by the original host. Alternatively, the employed *Tam3* copy is a mutated element with altered specificity.

Table 2. Activity of two-element systems *Ac/Ds*, *En/I(Spm/dSpm)* and *Tam3/dTam3* in heterologous plants. Activation of non-autonomous elements by an autonomous or immobilized transposase-producing element has been recorded by molecular (mol.) and phenotypic assays (NPTII, HPT, SPT and GUS). Excision frequency has been determined both in progeny of crosses (*) and in somatic tissues where one of the components is transformed into a plant already containing the other component. The efficiency of re-integration of a non-autonomous element into the host genome is indicated when measured.

Activator	Target	Assay	Excision freq.	Integration	Ref.
Ac/Ds					
<i>Nicotiana tabacum</i>					
Ac	AcΔ (3 kb)	NPTII	50%	ND	12
2'-Ac DNA	AcΔ (3 kb)	NPTII	ND	ND	12
Ac-18 ¹	AcΔ (3 kb)	NPTII	24–50% (*)	+	29
Ac	AcΔ (3 kb)	NPTII	50%	ND	45
Ac	AcΔ DHFR	NPTII	30–60%	+ (60% ²)	45
Ac	AcΔ GUS ³	HPT	45–50%	Rommens, unpubl.	
Ac ⁴	AcΔ (4.6 kb)	SPT	ND (*)	+	34
<i>Lycopersicon esculentum</i>					
Ac	Ds-1	mol.	100%	+	40
Ac	Ds 202 ⁵	mol.	ND	+	40
Ac	Ds 204 ⁶	mol.	0%	ND	40
En/I and Spm/dSpm					
<i>Nicotiana tabacum</i>					
En	I (2.2 kb)	mol.	ND	+	48
Spm	dSpm	GUS	52–56%	ND	44
35S-Spm	dSpm	GUS	39–55%	ND	44
Tam3/dTam3					
<i>Nicotiana tabacum</i>					
Tam3 ΔTIR	dTam3 ⁷	HPT	5%	16%	28

¹ Immobilized Ac, with a deletion of 4 bp from the 3' terminal inverted repeat (TIR).

² Phenotypic assay for re-integration: resistance to methotrexate, also molecular analysis of AcΔ DHFR (4.4 kb) activation in *Arabidopsis thaliana*.

³ AcΔ GUS contains the GUS gene and the plasmid pUC18 in the *Hind*III site (7.8 kb).

⁴ Ac modified at position 170 to create a *Cla*I site.

⁵ Ds 202 carries bacterial β-galactosidase gene in Ac sequence (7.4 kb).

⁶ Ds 204 carries β-galactosidase gene flanked by 5' 185 bp and 3' 131 bp of Ac (1.7 kb).

⁷ dTam3 is a 6 kb *Tam3* element carrying plasmid pACYC184.

Mutation analysis of transposable element *Ac* in tobacco

Because the maize transposable element *Ac* behaves in a similar in maize and heterologous plant species in the tested aspects, mutation analysis in heterologous plant species would allow the determination of functionally important regions of the transposable elements.

The first deletion analysis of the autonomous *Ac* element [12] indicated that not only intact terminal inverted repeats are required in *cis* for the

activity of *Ac*, but also subterminal regions close to the 5' end of the element (nucleotides 44–181). Deletion derivatives of *Ac* without these sequences can no longer transpose autonomously nor can they be *trans*-activated by an intact *Ac* element. For the action in *trans* the transposase (orfA) appears to be essential: deletions of nucleotides 950–1060 or 1783–3381 immobilize the *Ac* element, but the capacity to be *trans*-activated by an intact *Ac* element remains. Further experiments indicate that most of the long 5' untranslated leader (nucleotides 246–920) can be deleted

without abolishing transposition of *Ac*. The data of this deletion study are compatible with *in vitro* studies on the binding of the *Ac* orfA protein to fragments of the transposable element. Primarily subterminal regions of *Ac* bind the purified protein in a gel-retardation assay [38]. With *Bal*31 deletion studies the transposase binding sites were delineated to the area of 102–158 and 4450–4527 (Fig. 2), which also contains multiple copies of the AAACGG motif that can bind the orfA protein when the motif is supplied as an oligomer.

A detailed analysis in tobacco has delineated the *cis*-required regions of *Ac* accurately [13]. Both ends contain fragments that are important

for 'normal' transposition (nucleotides 186–238 and 4356–4435), while the areas close to the ends of the *Ac* element (overlapping the deletions described for the autonomous *Ac*) are essential and only respond to *trans*-activation at a frequency below 10% of the wild-type activity. Therefore the smallest *Ds* element that is still fully active would have to contain the nucleotides 5' 1–238 and 3' 4356–4565. The fact that neither two 5' nor 3' *Ac* fragments can be used to construct an active element suggests that there are different functions for both ends.

Ac inactivation is strongly correlated with methylation of sequences in the 5' untranslated leader [9, 18]. Alternatively, regulation of *Ac*

Fig 2. Functional properties of the 5' end and the 3' end of the *Ac* element from *Zea mays*. *In vitro* binding studies of purified *Ac* orfA protein delineated major binding areas (black boxes), that are located in CpG-rich, methylation-sensitive areas. Regions essential for transposition of *Ac* have been mapped by mutation analyses in transgenic tobacco plants. DNA segments that influence the efficiency of the transposition process may have regulatory functions. The putative start (334) and end (4302) of the mRNA encoding the transposase function (orfA) are indicated. Coordinates of the functional regions are relative to the 5' end of the *Ac* element. Inactivating deletions have rendered the *Ac* element immobile and unresponsive to *trans*-activation.

transposition could be achieved by methylation of the transposase binding sites. This form of regulation is feasible because the transposase-binding areas and the regions required in *cis* for transposition are rich in CpG motifs that are sensitive to methylation [39]. Furthermore, *in vitro* studies indicated that the purified 'transposase' has a higher affinity for hemi-methylated binding motifs [38], making it obvious that activation of *Ac* can be accomplished by methylation. However, methylation of the *Ac* element has not been studied in detail in heterologous hosts (except in tobacco, where methylation of *Hpa* II sites has not been detected [30, 57]), and needs to be analysed more accurately by genomic sequencing.

Some form of regulation of transposition is suggested by *orfA*-protein deletion studies in tobacco and petunia [32, 41]. The N-terminal 101 amino acids can be removed without significantly altering the capacity to *trans*-activate a resident *Ds* element. This truncated protein does not require the nucleotide 186–238 box (see above) for autonomous transposition. Therefore it can be envisaged that the N-terminal part of the *orfA* protein plays a role in the regulation of 'normal' transposition by an interaction with the 186–238 box. Further protein deletion studies may be used to determine the peptide domains that have the binding specificity for the ends of *Ac* and putative endonuclease domains. The *in vitro* binding of maize nuclear proteins to fragments at the 5' and 3' end of *Ac* close to the *Ac* *orfA* binding domain suggest that host factors may also play a role in the regulation of *Ac* transposition [38].

Analysis of the *En/Spm* suppressor function in tobacco

Although no functional analysis of the transposase function of *En/Spm* has been performed in transgenic plants, tobacco protoplasts were employed to analyse the suppressor function that has been assigned to the *En/Spm* element. It has been postulated that the transposase (*tnpA*) molecule(s) bind(s) to the ends of a *dSpm/I* element that is inserted into a gene involved in pigment

synthesis, thereby inhibiting expression of this trait [22, 51]. By inserting *En/Spm* fragments into the 5' and 3' untranslated region of a GUS reporter gene and combining these constructs with a transposase *tnpA* expressing vector the repressor function could be reconstituted [24]. Only the 5' and 3' ends of the *En/Spm* element can provide a target for the binding of the *tnpA* molecule that results in inhibition of GUS expression. The *in vitro* determined binding motif for the *tnpA* protein [22] exhibited this repressor-target function only when present as a tail-to-tail inverted repeat in the GUS reporter gene. The distribution of the binding motifs in the ends of the *Spm* element is such that these would promote binding of the transposase molecule when a stem loop structure is formed with both ends. Although it has been suggested that the *tnpB* gene product from *En/Spm* may represent the function that cleaves the ends of this element [21] no data have yet been obtained to confirm this. The regulation of *tnpA/tnpB* expression and the influence of methylation on the activity of *En/Spm* offer possibilities to study the transposition mechanism and the regulation thereof. The results of experiments with *Ac* and *Spm* clearly demonstrate the use of a transgenic system to dissect the transposition process.

Prospects of the application of heterologous mobile DNA elements in transposon tagging

Based on the results discussed above it appears to be possible to employ either an *Ac* or an *En/Spm* element for transposon tagging strategies in heterologous plant species. In maize the frequency of *Ac* or *En/Spm* induced mutations varies from 10^{-5} to 10^{-6} , when a transposable element is not genetically linked to the gene to be tagged [14]. When a transposable element is closely linked to the gene of interest, the frequency of insertions increases to $10^{-3} - 10^{-4}$ [14]. This observation can be explained by the assumption that both *Ac* and *En/Spm* preferentially transpose to linked sites [14, 26, 8].

It is therefore essential to know whether maize

transposable elements also transpose to linked sites in a new genetic background. Genetic experiments in tobacco indicate that transposed *Ac* copies are predominantly inserted within two map units from the original site (the SPT marker gene) [34]. Only 3 copies out of 14 tested have inserted at unlinked sites. This experiment is the first genetic analysis indicating that *Ac* also preferentially transposes to linked sites in the tobacco genome. Molecular analysis of tomato progeny suggested that in half of the primary transformants analysed transposition occurred to linked sites [4]. For insertional mutagenesis it would be advantageous if a transposable element inserts into unique DNA and not preferentially into repetitive sequences. The flanking DNA sequences of new *Ac* and *En/Spm* insertions in the tobacco and potato genome were characterized by DNA gel blot analyses [1, 20, 30, 48]. Both repetitive (3 cases) and low copy number DNA (5 cases) were found to flank *Ac* inserts, while *En* insertions were flanked by unique DNA sequences (three cases) and middle repetitive DNA (one case). The results suggest that *Ac* and *En/Spm* integrate more often into low copy DNA.

The tagging of a specific plant gene in a heterologous host should be possible with an autonomous transposable element. Because of the higher frequency of linked transpositions it will be desirable to locate the transposon tag close to the locus that contains the gene of interest. A transposable element that is linked to this gene can be selected from a stock of primary transformants, with a combination of the IPCR technique and RFLP mapping: the plant DNA sequences flanking the non-autonomous element can be amplified by the IPCR technique (inverse PCR [16]) and used as a probe in RFLP analysis of a characterized population of progeny (suitable for plants like tomato and *Arabidopsis* [7, 56]). Employment of an autonomous *Ac* for the purpose of gene-tagging would mean that one can profit from the higher activity of *Ac* in a homozygous state, increasing the possibility of an insertion into the target gene. In addition, it ensures that the *Ac* element will not excise frequently from the target gene because it is less active in a heterozygous state [35, 36]. This

property will make it easier to detect a mutated phenotype, as it will reduce variegation. The advantage of this approach to tag a gene is that within two generations screening of mutant phenotypes becomes possible. On the other hand, the use of a two-element system is a good alternative, because of the possibility to control the transposition process. As the non-autonomous element (*Ds* or *I/dSpm*) is immobile it will remain at the chromosomal position where it has been inserted after transformation. Following selection of a transformant which contains the non-autonomous element linked to the target gene and after combination with an immobilized activator element by a second transformation event or a cross, the non-autonomous element can be induced to transpose and mutant selection can be initiated (Fig. 3). Because the plants to be

Fig 3. A directed transposon tagging strategy. A non-autonomous element equipped with a genetic marker and recloning facilities is introduced into a heterologous plant species. A single copy that is linked to the gene of interest (R) is selected. A homozygous plant with this characteristic is crossed with a plant recessive for the trait (r) and homozygous for an inducible activator element. All progeny will be heterozygous for the trait (Rr) and for the non-autonomous / activator elements, thus allowing transposition to occur. Mutant or variegated progeny can be selected and analysed for linkage to the genetic marker of the non-autonomous element. The tagged gene R' can now be recloned using the facilities of the non-autonomous element.

screened will be chimaeric for the mutated phenotype the trait that is examined should preferably be expressed in a cell-autonomous way (compare the results with the phenotypic assays employing cell autonomous marker genes [19, 36, 54]).

To obtain as many independent insertions as possible of the *Ds* or *I/Spm* element in the progeny of such a plant it would be helpful if the activity and the timing of transposition could be regulated. This might be achieved by regulating the expression of the transposase function. The cDNA coding for *Ac*-orfA (transposase) driven by the T-DNA 2' promotor can *trans*-activate *Ds* from the NPTII reporter gene indicating that expression of this function is sufficient to carry out the transposition process [12]. Experiments with *Ac* activator elements containing different promoters have been initiated in several laboratories to examine the influence of promoter strength and specificity on the timing and frequency of *trans*-activation of *Ds*. The CaMV35S, *ocs* and *nos* promoters were fused to the *Ac* transposase and compared for excision timing during cotyledon development using an SPT:*Ds* assay. The *Ac* transposase fusion with the CaMV35S promoter gave large green sectors indicating early excision of *Ds* from the SPT gene, the *ocs* fusion gave small sectors and the *nos* fusion very small sectors. As yet no information is available on germinal excision brought about by these constructs (S. Scofield and J. Jones, unpublished). The same promoter-*Ac* transposase fusions were used to monitor *Ds* activation from the SPT gene in *Arabidopsis*. While the *nos* *Ac* transposase fusion was incapable of inducing green sectors (*Ds* excision) in cotyledons of F1 seedlings, the *Ac*, *ocs* and CaMV35S promoter fusions all induced variegation. Expression of the *Ac* transposase by the *Ac* or *ocs* promoter was only sufficient for *Ds* activation in some of the progeny, while the stronger CaMV35S promoter induced variegation in all seedlings harbouring both elements. In the F2 progeny of variegated F1 plants harbouring the *ocs* promoter fusions no full revertants (green seedlings) were detected and only 1% of the seedlings were variegated, indicating inactivation of the *ocs* promoter in the next

generation. Although some inhibiting effect on the transposase expression by the CaMV35S promoter was visible as a lower frequency of green sectors in F2 seedlings, the progeny of most F1 plants included many germinal revertants. The stronger CaMV35S promoter can therefore overcome the inactivation in the next generation (G. Coupland, unpublished). therefore it can be concluded that promoter manipulation can influence the rate of *Ac* transposition in tobacco and *Arabidopsis*. However, the observation that transposition can be reduced in the next generations has to be taken in to account when developing transposon tagging strategies for *Arabidopsis* and other plant species. A more pronounced effect on the timing of transposition may be obtained by the employment of inducible or pollen-specific promoters that will induce transposition in independent gametes. The *Arabidopsis* ADH promoter [6] and the *Petunia* CHI-A2 promoter [60] have been selected for this purpose, but no definite results have yet been reported.

Not only the activator element can be manipulated to optimize the transposon tagging procedure. The non-autonomous element can be equipped with a genetic marker to be able to confirm directly that induced mutations are linked to the genetic marker. To facilitate recloning of tagged genes the non-autonomous element can be equipped with bacterial cloning vectors. It has been demonstrated that a genetic marker, the DHFR gene that makes a plant resistant to methotrexate, can be fit into a *Ds* element without significantly altering the capacity to be *trans*-activated [45] (see Table 2). When a *Ds* element is equipped with a GUS reporter gene and a bacterial plasmid it can still be *trans*-activated by *Ac* and reintegrates efficiently (unpublished results, C. Rommens). In addition, other applications for manipulated non-autonomous transposable elements can be proposed. When such an element is equipped with a strong promoter, random insertion of this element might result in the overexpression of a gene it inserted next to. Especially regulatory genes with an effect on plant morphology might be detected and cloned with this strategy. Alternatively, a non-autonomous trans-

posable element can be equipped with a reporter gene without promoter sequences. Upon integration close to a plant promoter the expression is restored. In this way random isolation of plant promoters should be possible. Finally, results with a transient excision assay for a *Ds* element in *Petunia* [32] suggest that non-autonomous transposable elements may be developed into gene-transfer vehicles, similar to the *Drosophila* P-element system and as an alternative for *Agrobacterium* mediated T-DNA transfer.

As many laboratories have set out to develop a transposon tagging strategy for heterologous plant species (i.e. tomato and *Arabidopsis*), it is soon to be expected that genes will be isolated by this method. A documented effort towards the cloning of a tobacco gene is the employment of an autonomous *Ac* element in the search for the TMV resistance gene *N* [31]. Approximately 40 000 F1 seedlings were analysed from a cross between *Nicotiana tabacum* cv. Samsun NN (transformed with *Ac*) and *Nicotiana tabacum* cv. SR1 nn. *Ac* insertion into the *N* gene should lead to loss of the hypersensitive response and result in systemic TMV infection of tobacco. Several of the 30 systemically infected plants recovered from this experiment displayed patches of necrotic lesions that suggest somatic instability of the mutation. None of these plants carried an *Ac* element linked to the mutant phenotype, suggesting in fact that these are spontaneous mutants. We conclude that one has to have some idea about the spontaneous mutations in the target gene and care has to be taken with mutants arising through somaclonal variation during the transformation procedure [63]. Moreover it might be preferable to use genetically linked transposon tags, because a tagging strategy with a transposon tag that is not linked to the gene of interest will involve large plant populations to be screened.

In order to develop a general method for the cloning of genes without known products efforts will have to be made to generate and map as many independent insertions of the transposon tag as possible. Lines that carry the transposon tag linked to the gene of interest can then be selected to initiate a mutant selection procedure.

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