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Published in:
 Plant Molecular Biology

DOI:
[10.1007/BF00017915](https://doi.org/10.1007/BF00017915)

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Document Version
 Publisher's PDF, also known as Version of record

Publication date:
 1991

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Haring, M. A., Teeuwen-de Vroomen, M. J., Nijkamp, H. J. J., & Hille, J. (1991). Trans-activation of an artificial dTam3 transposable element in transgenic tobacco plants. *Plant Molecular Biology*, 16(1).
<https://doi.org/10.1007/BF00017915>

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Trans-activation of an artificial dTam3 transposable element in transgenic tobacco plants

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Received 26 June 1990; accepted in revised form 11 September 1990

Key words: transposition, transposon tagging, two-element system, Tam3 rearrangements, transgenic plants, phenotypic assay

Abstract

In *Antirrhinum majus* only autonomous Tam3 transposons have been characterized. We investigated whether an artificial dTam3 element, with a deletion in the presumptive transposase coding region, can be *trans*-activated in tobacco by an activator Tam3 element, which was immobilized by the deletion of one inverted repeat. A phenotypic assay based on restored hygromycin resistance demonstrates that a dTam3 element harbouring a bacterial plasmid can be *trans*-activated with a low frequency. Molecular analysis confirms that the dTam3 element has been excised from the HPTII marker gene. Reintegration of the dTam3 element into the tobacco genome is detected only in one out of six hygromycin-resistant plants analysed. PCR analysis of empty donor sites shows that excision of the dTam3 element in tobacco results in rearrangements (deletions and additions), that have been shown to be characteristic of Tam3 excision in the original host *Antirrhinum majus*. This *trans*-activation assay allowed us to establish that, in contrast to what has been detected in *Antirrhinum majus*, a periodical temperature shift down to 15 °C does not enhance dTam3 transposition in regenerating tobacco calli.

Introduction

Although many plant genes have been cloned with conventional recombinant DNA technology based on the knowledge of the gene product, many genetically well described traits cannot be cloned because the gene product has not been characterized biochemically. For these traits transposon tagging can be a biological tool for the characterization of the gene as demonstrated for several maize and *Antirrhinum* genes [5, 12, 16, 17]. These plant species harbour several genetically and molecularly characterized transposable elements that can be employed for this purpose. However, for most plant species no transposons

have been characterized. Still, in those species accessible to genetic manipulation, the well characterized Ac and Tam3 elements can be introduced to enable transposon tagging. Both elements have been shown to transpose in transgenic plants [1, 6, 9, 22, 23].

For the purpose of controlled transposon tagging it is advantageous to employ a two-element system, in which the activator is immobilized by deletion of the inverted repeat but provides a *trans*-acting factor that induces transposition of the target element, that has inverted repeats but a deletion in the transposase function. The target element can be equipped with a marker gene and cloning vector to facilitate localization

and recloning from the plant genome. When independent and stable T-DNA inserts have been generated in a transgenic plant, these can be combined with an immobile activator that is regulated to give a frequent and correctly timed *trans*-activation. The Ac/Ds system of *Zea mays* is an example of a naturally occurring two-element system that can be used for the purpose of transposon tagging [17]. *Trans*-activation of the maize Ds element in transgenic tobacco plants has been demonstrated both by transformation [4, 7, 15] and by crosses that introduce Ac [7, 10]. Although autonomous Tam3 transposition occurs both in *Antirrhinum* and transgenic plants [6, 13], *trans*-activation of a dTam3 element has not yet been demonstrated. Nevertheless, it may be advantageous to develop a two-element system for Tam3 in heterologous plant species, to enable controlled transposition. Since Tam3 transposition has been shown to be inducible by temperature down-shift in its original host [2, 12], temperature treatment might be a further tool exerting such control.

The development of a phenotypic assay, employing several marker genes, has resulted in a sensitive method to establish excision of a transposable element [1, 6, 8, 21]. When the transposon excises from the leader of the HPT II marker gene, a hygromycin-resistant phenotype is obtained [6]. It should therefore be possible to register the *trans*-activation from the HPT II marker gene of a dTam3 element with intact inverted repeats but a deletion in the transposase coding region, when it is combined, within one cell, with an immobilized activator, Tam3 Δ TIR. In this paper we describe the first evidence for *trans*-activation of a 5.7 kb artificial dTam3 element in transgenic tobacco plants. The properties of this Tam3 two-element system will be compared with the activity of the Ac/Ds system in tobacco. As Tam3 excision in tobacco appears to cause rearrangements similar to those described in *Antirrhinum majus*, we will discuss the possibilities for an artificial Tam3 two-element system in the analysis of factors involved in the transposition process.

Materials and methods

Plant material and transformation

Transgenic tobacco plants that harboured intact T-DNA copies of either pTT21806 (Tam3 Δ TIR) or pTT21840 (dTam3, Δ transposase) [6], were selected by Southern blot analysis. Plant numbers 2001, 2002 and 2008 contained pTT21806 while plant number 2105 harboured pTT21840. Greenhouse-grown plants were transformed with *Agrobacterium* strains as described [6]. Selection was carried out on medium containing vancomycin (200 mg/l) and 20 mg/l hygromycin B during the first two weeks and the same medium but 40 mg/l hygromycin B during the following period.

The temperature induction experiment was carried out as the transformations described above, but half of the treated leaf discs were incubated at 15 °C, in the light, for six hours every day.

DNA isolation, Southern blot analysis

DNA was isolated from greenhouse-grown plants as described [6]. 10–15 μ g DNA was digested with 50–80 units restriction enzyme (Bethesda Research Laboratories, BRL) and agarose gels were blotted onto Hybond N⁺ by vacuum blotting (LKB). Hybridization with random priming labelled probes was carried out in 10% Dextrane sulphate (Pharmacia), 1 M NaCl, 1% SDS and 200 μ g/ml denatured herring sperm DNA at 60 °C. After washing down to 0.1 $\times$ SSC at 60 °C blots were autoradiographed using Kodak X-Omat AR films.

PCR analysis

The polymerase chain reaction [19] was performed in 100 μ l containing 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, 0.05% Tween 20 and 200 μ M dNTPs. For amplification of the empty donor site

100 pmol of a CaMV 35S primer (5' ATATCT-CCACTGACGTAAGGGATGACG3') and a HPTII primer (5' GAATTCCCCAATGTCAA-GCACTTCCG3') were added to 1 µg plant DNA together with 1 unit Taq polymerase (Ampli-Taq., Perkin Elmer Cetus) and 30 cycles were performed: denaturation 94 °C 20 s, annealing 55 °C 20 s, extension 72 °C 1 min. Fragments were separated on a 2% agarose gel.

Results

Phenotypic assay for trans-activation

Tam3 derivatives with a deleted terminal inverted repeat (Tam3ΔTIR, the immobilized activator) or with a deletion in the transposase coding region (dTam3, the target element) have been shown to be unable to transpose in transgenic tobacco plants [6]. Combining these two Tam3 elements within one cell by a second transformation event should allow us to establish *trans*-activation of the dTam3 copy.

Tobacco plants containing T-DNA from pTT21806 (Tam3ΔTIR) were transformed with an *Agrobacterium* strain containing plasmid pTT21840 (dTam3 cloned in the leader of the HPT II gene [6]). After two days of incubation with *Agrobacterium* the leaf discs were put on selective medium containing 20 mg/l hygromycin. Although callus growth progressed slowly on all leaf discs only some calli gave rise to green shoots. These shoots were rooted and leaves were tested for their ability to form shoots on medium containing 40 mg/l hygromycin. The resistant phenotype is clearly distinguishable from the parental plant phenotype (Fig. 1) and can only be contributed by the restoration of the HPTII gene expression by excision of the dTam3 element. No spontaneous activation of the dTam3 element has been recorded in tobacco plants without an activator Tam3 element, so we presume that excision of the dTam3 element results from *trans*-activation by the Tam3ΔTIR element. Because the first resistant shoots arise eight weeks after the transformation event we have to assume that the

Fig. 1. Phenotypic assay for *trans*-activation of dTam3. Tobacco leaf explants are grown on regeneration medium containing hygromycin (40 mg/l) for 3 weeks. The activator plant 2001 (A) is sensitive to hygromycin. After a second transformation event with an *Agrobacterium* strain harbouring pTT21840 (dTam3) plants can be obtained that are hygromycin-resistant, for example plant 4202 (B).

Tam3 *trans*-activation does not occur early in the developing callus. The number of hygromycin-resistant transformants was similar for the three plants tested (Table 1). No hygromycin-resistant shoots were detected on non-transformed leaf discs.

The reverse experiment was carried out by transforming leaf discs from a plant containing pTT21840 (dTam3), with an *Agrobacterium* strain harbouring pTT21808 (Tam3ΔTIR). This transformation experiment yielded a comparable number of shoots resistant to hygromycin as in the previous experiments. No hygromycin-resistant shoots were detected on non-transformed leaf discs nor on leaf discs transformed with pBIN19 (a vector without Tam3 activator sequences). These results indicate that *trans*-activation of dTam3 from the HPTII gene of pTT21840 is possible and will only occur in the presence of a Tam3 activator element.

Molecular analysis of excision and reintegration of the dTam3 element

To confirm the excision of the dTam3 element from the marker gene molecularly, DNA of hygromycin-resistant plants was isolated and digested

Table 1. Phenotypic assay for *trans*-activation of dTam3.

Plant number	<i>Agrobacterium</i> strain harbouring plasmid	Number of leaf discs tested	Number of Hyg ^r shoots
Tam3ΔTIR plants			
2001	–	200	0
2001	pTT21840	105	4
2002	pTT21840	107	5
2008	pTT21840	81	5
dTam3 plant			
2105	–	100	0
2105	pTT21808	74	5
2105	pBIN19	86	0

Leaf discs of transgenic tobacco plants were transformed with an *Agrobacterium* strain harbouring either the target dTam3 element (pTT21840) or the activator Tam3 element (pTT21808). Shoots arising on medium containing 40 mg/l hygromycin indicate *trans*-activation of the dTam3 element from the HPTII gene.

with *Xba* I (Fig. 2 part A). The original plant (Tam3ΔTIR, No. 2001) yields a 1.3 kb fragment when hybridized with the HPTII probe. In the hygromycin-resistant tobacco plants, in which the dTam3 element has been excised from the HPTII gene (4202 and 4203), an extra, 1.35 kb band is visible: the empty donor site fragment. Plant 4202 also carries a 2.4 kb band which indicates the presence of a non-transposed dTam3 element as well. When this filter is reprobed with Tam3 the parental plant reveals three bands: the internal 2.6 kb band and two border fragments (3 kb and 6 kb) characteristic of the T-DNA insertions (at least two copies) in this plant. While plant 4203 exhibits this same pattern, new bands appear in the DNA of plant 4202. These new bands indicate the presence of the dTam3 element in this plant, the 3.9 kb fragment representing an internal fragment and the 2.4 and 0.9 kb bands resulting from non-transposed dTam3 copies. However two new bands (3.5 kb and 10 kb) appear in this lane suggesting integration of the transposed dTam3 element.

Direct evidence comes from the hybridization of *Eco*RV digested DNA from the parent plant and plant 4202 with HPT, Tam3 and pACYC184

respectively (Fig. 2 part B). Hybridization with the HPTII probe lights up two new bands (6.6 kb and 8 kb) in plant 4202, apart from the 4.0 and 3.2 kb parental bands (lane 8). These two bands (6.6 kb and 8 kb) hybridize with Tam3 as well, but a third band is detected with the Tam3 probe (lane 10). While the 4 kb, 1.6 kb and 1.2 kb bands are present in both the parental plant and plant 4202, a 6.3 kb fragment is detected with Tam3 hybridization and not with the HPTII probe. Because the dTam3 element does not contain an *Eco*RV site these three fragments represent individual dTam3 copies. The observation that one of these dTam3 copies is no longer inserted into the HPTII gene proves that it has integrated elsewhere in the tobacco genome. To confirm that these three Tam3 fragments have not arisen from some unexplainable rearrangement of the parental DNA we hybridized the same blot with pACYC184. Although some background bands are visible in both lanes the three Tam3 bands are shown to contain this plasmid (Fig. 2 lanes 11 and 12) and therefore can only be designated dTam3 copies. For plant 4203 and four other plants examined no integration of a dTam3 copy could be established (results not shown).

Fig. 2. Excision and reintegration of a *trans*-activated dTam3 element.

A. Excision of an introduced dTam3 element has been analysed by Southern blot analysis of *Xba*I digested DNA from the parental plant (P, No. 2001) and two hygromycin-resistant plants 4202 and 4203. Asterisks indicate new Tam3 fragments, the 1.3 kb fragment visible in lane 4, 5 and 6 is due to incomplete removal of the HPTII probe, prior to reprobing with Tam3.

B. Integration of a dTam3 element into the DNA of plant 4202 is revealed by hybridization of the *Eco*RV digest of the parental plant (P) and plant 4202. Probes used are indicated above the lanes. The transposed copy of dTam3 is indicated by an arrow. Fragment sizes are in kilobase pairs (kb).

C. Partial physical map of the parental plant (P, No. 2001) DNA and the introduced dTam3 element before and after excision of the dTam3 element from the HPT II gene. cmv stands for the CaMV 35S promoter fragment. The HPTII probe consists of a 1 kb fragment from the HPTII gene, while the Tam3 probe consists of a 3.6 kb Tam3 fragment from plasmid pVUT22 [6]. Probe fragments are underlined.

A polymerase chain reaction assay detects rearrangements of the empty donor site

When excision of the dTam3 element occurs the CaMV 35S-HPTII gene construct is restored. Applying the polymerase chain reaction [19] with primers adjacent to the empty donor site to DNA from a hygromycin-resistant plant should reveal the excision of the dTam3 element. With a CaMV 35S primer and a HPTII primer a 380 bp fragment should be synthesized on the unchanged pTT218 template. A fragment produced by a perfect excision of Tam3 should be 62 bp longer than the original fragment because the *Bam* HI cassette used for cloning the dTam3 element in the marker gene still contains extra sequences. Excision products are expected only in DNA samples from hygromycin-resistant plants, not in the parental plant DNA, because the size of this fragment, 6.0 kb, prevents it from being amplified with the

thermal cycle employed (Fig. 3, part A). We analysed the DNA of hygromycin-resistant plants with this PCR method and detected a fragment of approximately 440 bp in 8 plants. An example of the analysis of this group of plants is shown in Fig. 3 part B, lane 2. Five other hygromycin-resistant plants yielded fragments different from the expected size (Fig. 3 lanes 3, 4, 6, 7, 8). The rearrangements of the empty donor site fragment include both deletions: - 40 (Fig. 3 lane 4), - 20 (lane 6) and additions + 20 (lane 7), + 60 (lane 8) and + 300 (lane 3). If the fragments of 440 bp represent perfect excisions, it can be concluded that during *trans*-activation of a dTam3 element under the described conditions, in transgenic plants, perfect and aberrant excision takes place with a comparable frequency. The changes in the empty donor site fragments due to Tam3 excision are similar to the variations found in the original host *Antirrhinum majus* [3, 20].

Fig. 3. PCR analysis of dTam3 excision from the HPTII gene reveals rearrangements of the empty donor site fragment. Part A gives a schematic representation of the expected fragments produced on the intact CaMV 35S-HPTII gene template (pTT218), an excision product and the parental DNA. Part B shows a selection of the analysed hygromycin-resistant plants and products produced on parental DNA (lane 1) and pTT218 (lane 5). Products of the polymerase chain reaction have been run on 2% agarose gel. Size references are in basepairs (bp). Lane 1: parental plant 2001, lane 2: expected empty donor site plant 4002, lane 3: plant 4004, lane 4: plant 3902, lane 5: intact CaMV 35S-HPTII gene from pTT218, lane 6: plant 3903, lane 7: plant 4202, lane 8: plant 4203. Plant numbers represent individual hygromycin-resistant plants obtained in the *trans*-activation experiments.

Temperature induction of dTam3 trans-activation

As Tam3 transposition in *Antirrhinum majus* exhibits the capacity to be induced up to 1000-fold by a temperature shift from 25 °C to 15 °C, we tried to determine whether this capacity is specific for the Tam3 element and can be reproduced in transgenic tobacco plants. Leaf discs of a plant harbouring the dTam3 element in the HPTII gene (plant 2105), were transformed with *Agrobacterium* strains harbouring the activator Tam3 Δ TIR element. We recovered 3 hygromycin-resistant shoots from the 183 transformed leaf discs grown at 25 °C and 2 hygromycin-resistant shoots from the 103 transformed leaf discs that were cultured at 15 °C, six hours every day, for three weeks. These results suggest that there is no effect of the 15 °C treatment on the Tam3 *trans*-activation in regenerating tobacco calli.

Discussion

Although Tam3 transposition has been characterized in detail both in *Antirrhinum majus* [3, 12, 20] and in transgenic tobacco plants [6, 13], no two-element system of this transposon has yet been described. We demonstrate that the activation of an artificial dTam3 element can be achieved by combining it with an immobilized transposase donor element: Tam3 Δ TIR. Replacement of a 1.4 kb internal *Cla*I-*Nco*I fragment by plasmid pACYC184 produces a non-autonomous Tam3 element that still can be activated by Tam3 transposase. The frequency of *trans*-activation in this system measures approximately 5%, when we assume a transformation efficiency of one transformant per leaf disc. An autonomous Tam3 element is excised in 20–40% of the tobacco calli analysed by a similar phenotypic assay [6]. With the phenotypic assay we employed, this Tam3 two-element system appears to be at least 4–8 times less active than the autonomous element. Although a reduction of the efficiency is also detected in the Ac/Ds system [4, 10], this two-element system almost retains the activity of the autonomous element. The rea-

son for the lowered Tam3 activity may be found in the extra 3.7 kb plasmid DNA that this dTam3 element has to carry. Preliminary data suggest that this may be the reason because a dTam3 element without this extra sequence is *trans*-activated with a higher frequency (M. Haring, in preparation).

Southern blot analysis shows that the expected empty donor site fragments are produced upon activation of the dTam3 element. However more accurate analysis of excision, applying the polymerase chain reaction, indicates that the dTam3 element has caused rearrangements that change the empty donor site fragment. Both deletion and addition of DNA segments can be seen in half of the analysed plants. Size rearrangements due to Tam3 excision have been reported in *Antirrhinum majus* [11, 14, 18, 20]. These rearrangements are thought to occur as a result of the excision mechanism of Tam3 [3]. Therefore we can conclude that the rearrangements caused by excision of Tam3 are comparable in the homologous and heterologous system and thus can be interpreted as a property of the transposition mechanism of Tam3. An additional consequence of these rearrangements caused by dTam3 excision may be the deletion of part of the HPTII gene, or larger additions that prevent expression of the gene. This may account for a lower excision frequency determined by the phenotypic assay than the actual excision frequency.

Following excision from the HPTII gene of plant 4202 the dTam3 element could be demonstrated to integrate at a new position in the tobacco genome. In five other cases we were unable to detect an integrated dTam3 copy. Although an artificial Ds element was lost after excision from a marker gene upon *trans*-activation by Ac, 60% of the tobacco calli retained the element [15]. Therefore, Tam3 seems to be less efficient in completing the transposition process in *Nicotiana tabacum* than Ac. As already suggested by Martin *et al.* [13] the integration of Tam3 may involve host factors that supplement the transposase function, factors which are absent or different in the new host, tobacco. The *Zea mays* Ac element seems to be able to function properly in

this genetic background [1, 7] and might therefore be regarded as less related to Tam3 than has been suggested before [3].

One of the most striking features of Tam3 is the temperature-enhanced excision recorded at independent loci in *Antirrhinum majus* [2, 12]. Although these data suggest that it is an element-specific property no enhancement of Tam3 excision could be demonstrated in tobacco plants [13]. We tested the effect of a temperature downshift on the ability of a newly introduced Tam3 Δ TIR copy to activate a dTam3 element residing in a transgenic tobacco plant. The frequency of activation did not differ significantly between leaf discs grown at 15 °C for 6 hours every day and leaf discs grown at 25 °C during the selection period. This suggests that temperature-enhanced excision is not encoded by the Tam3 element itself but that host factors like the described Stabilizer gene [3, 12, 13] may be responsible for this temperature effect in *Antirrhinum majus*.

With regard to the potentials of the Tam3 two-element system for transposon tagging, we conclude that the relatively low excision and integration frequency compared to the Ac/Ds system and the lack of temperature-induced transposition make it at the moment less suitable for gene tagging in transgenic plants.

The two-element system for Tam3 we describe here will allow further investigation of factors involved in the regulation of the transposition process. Deletion studies, as described for Ac [4], can be carried out to establish which parts of the transposon are essential as target for the transposase. Promoter manipulation of the putative Tam3 transposase can be carried out to investigate whether the level of expression influences the efficiency of the transposition process. Finally, more detailed analyses of Tam3 induced deletions and rearrangements will be possible in transgenic plants since no other Tam3 copies are present [3, 14]. These *trans*-activation studies will provide information on the transposition process of Tam3 and can help to elucidate which host factors are involved both in the excision of Tam3 and the reintegration into the plant genome. The com-

parison of Tam3 and Ac will allow us to determine what factors are involved in the different behaviour of these elements in a new genetic background.

Acknowledgements

The authors wish to thank Jonathan Jones and Colwyn Thomas, Sainsbury Laboratories, John Innes, Norwich for their help and advice with the PCR analysis. Anton Gerats is acknowledged for his critical review of the manuscript and Hansje Bartelson for typing the manuscript. M.H. was financially supported by the Dutch Programme Committee for Biotechnology.

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