Fusion of the transposase with a classical nuclear localization signal to increase the transposition efficiency of Ac transposon

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Abstract. A new strategy was applied to improve the transposition efficiency of the maize transposon Activator (Ac) in heterologous plants. The Ac transposase was fused with a classical nuclear localization signal (NLS) of SV40 to promote the transport of transposase into a nucleus. Base on this, two NLS-TPase constructs were yielded, one containing the full length transposase gene (termed as SV40TPase), the other containing the truncated transposase gene (lacking its first NLS-like signal, termed as SV39TPase). These two NLS-TPase genes were expressed in transgenic tobacco plants under the control of PR-1a promoter. Excision of non-autonomous transposable element (Ds) from luciferase (LUC) reporter gene constructs was employed to analyze the induction of Ac transposase containing NLS. Applying the LUC assay and PCR analysis, these new NLS-TPase sources triggered higher Ds excision efficiencies then the native transposase. Furthermore, the SV40TPase showed more ability then the SV39TPase to trigger the Ds element. The usage of this new inducible transposon for plant functional genomics is discussed.

Keywords: Ac transposase; Inducible transposon; Luciferase reporter gene; Nuclear localization signal.

Abbreviations: HPT, hygromycin phosphotransferase; NLS, Nuclear localization signal; NPT, neomycin phosphotransferase; LUC, luciferase; SA, salicylic acid; TPase, transposase.

Introduction

The maize transposon Activator (Ac) is an autonomous transposable element with a size of 4565 bp. It codes for a single gene product, the transposase (TPase), which together with the inverted repeats and about 300 bp from each end of the transposon and putative host factors is the only prerequisite for transposition of the Ac element in plants. The Ac element is active in a wide range of plant species, including several members of the Solanaceae, and in rice, carrot and Arabidopsis (Becker et al., 1986; Van Sluys et al., 1987; Knapp et al., 1988; Yoder et al., 1988; Houba-Herin et al., 1990; Izawa et al., 1991), and has proven to be a powerful genetic tool for yielding knockout mutants for plant functional genomic studies (for a review, see Haring et al., 1991). Recently, the International Rice Genome Sequencing Project (IRGSP) completed its sequencing of the entire rice (Oryza sativa) genome. Various strategies, including transposon tagging, have been used to produce a large population of mutant plants adequately assigning function to the abundant sequence information (for review see Jeon and An, 2001). However, using transposon as a tool to create knockout mutants in plants with large genomes, seems to require increased transposition efficiency. To this end, Scofield et al. have fused the Ac TPase with the cauliflower mosaic virus (CaMV) 35S RNA promoter and found, in tobacco, no direct proportionality between the amounts of TPase mRNA and Ac/Ds transposition activity (Scofield et al., 1992). Furthermore, transpositions occur only at TPase transcription level, below a critical threshold (Scofield et al., 1993). One possible explanation is that above this threshold the TPase starts to aggregate and transpositions cease (Heinlein et al., 1994), forcing us to consider another approach to improving transposition efficiency.

In principle, in order to perform the transposition events, the TPase can be transported into the nuclei, a process mediated by specific signals called nuclear localization sequences (NLSs) (Stochaj and Silver, 1992a). The TPase has three NLSs near its amino-terminal end, NLS (44-62), NLS (159-178), and NLS (174-206), each of which is sufficient to direct GUS to the nucleus (Boehm et al., 1995). However, transpositions occur only at TPase transcription level, below a critical threshold (Scofield et al., 1993). One possible explanation is that above this threshold the TPase starts to aggregate and transpositions cease (Heinlein et al., 1994), forcing us to consider another approach to improving transposition efficiency.
To date, the nuclear targeting signals of more than 70 mammalian and yeast proteins have been characterized (Dingwall and Laskey, 1991; Garcia-Bustos et al., 1991). Studies indicate that the nuclear transport machinery is highly conserved between animals, yeast and plants (Nelson and Silver, 1989; van der Krol and Chua, 1991; Lassner et al., 1991; Stochaj and Silver, 1992b; Hicks and Raikhel, 1993; Wagner and Hall, 1993). Three different categories of NLSs were identified. The SV40 large-T antigen NLS (ppKKKRKv) is the prototype NLS category, characterized by a short uninterrupted stretch of basic amino acids (Kaldéron et al., 1984; Lanford and Butel, 1984). The second most common category is the bipartite NLSs, consisting of two clusters of basic residues separated by a spacer peptide. The paradigm for the bipartite class is the Xenopus laevis nucleoporin NLS (KRpaatkkagqaKKKKI) (Dingwall and Laskey, 1991). The third category is represented by the yeast MAT alpha-2 NLS (KipiK) and consisting only one or two basic residues contiguous to hydrophobic amino acids (Hall et al., 1984).

Accordingly, we decided to use the SV40 large-T antigen NLS (ppKKKRKv) sequence to fuse with the TPase gene. Based on the inducible transposon system, the prototypic NLS of SV40 containing TPase was used as the source to trigger the transposition of the non-autonomous Ds element. We found that the NLS-containing TPase sources triggered higher Ds transposition efficiencies than did the native TPase source. Furthermore, it has been reported that the N-terminally truncated TPase derivative is inefficiently transported into the nucleus and aggregates predominantly in the cytoplasm (Heinlein et al., 1994). We constructed a similarly truncated TPase lacking the first NLS-like sequence of the native TPase. This construct was then fused with the prototypic NLS of SV40. We found that this truncated TPase derivative triggered slightly lower Ds transposition efficiency than did the NLS-containing full length TPase. The role of the NLSs for the transport of the TPase and the subsequent transposition was discussed.

Materials and Methods

DNA Manipulation

Recombinant DNA technology was performed according to Sambrook et al. (1989). The materials and methods required for the construction of plasmids pBC SK+ and pBinHygTs have been previously reported (Charng et al., 1995).

In order to generate modified NLSs constructs, which consist of the NLS of the SV40, two DNA fragments were yielded by polymerase chain reactions. To this end, the following synthetic oligonucleotide primers were used: primer SV39 (harboring 18 nucleotides coding for NLS and the remaining nucleotide identical to the Ac sequence from position 1404 to 1416, 5'-GGATCCATGAAGAAGAAGCGCAAAAGCTATTGTATCAGT-3'); primer SV40 (harboring 18 nucleotides coding for NLS and the remaining nucleotide identical to the Ac sequence from position 990 to 1104, 5'-GGATCCATGAAGAAGAAGCGCAAAAGCTATTGTATCAGT-3'), and primer CSV (complementary to the Ac sequence from position 1845 to 1823, 5'-AGTACTCATGTTCGAGACGCTTCCGGTGG-3'). Each polymerase chain reaction contained approximately 0.1µg template DNA (plasmid pBinHygTs), 0.25 µg of each primer, 0.2 mM dNTPs, 1.5 mM MgCl2, 1 unit of Taq DNA polymerase, and 10X buffer. The samples were subjected to 40 cycles of amplification with each cycle consisting of 1 min at 94°C, 30 s at 50°C, and 30 at 72°C. After reactions an agarose gel electrophoresis was performed to recover the DNA fragments. Each DNA fragment was then ligated with a 3.4 kb Smal fragment of plasmid pBC SK+. Then a Nsi I excised 1.7 kb TPase fragment from the pPCV7200RF was ligated to the Nsi I treated pBCSV40 and pBCSV39 vector, yielding the plasmids pLC40TP and pLC39TP. These two plasmids were digested with Bam HI, yielding the 2.5 kb fragment. This fragment was then ligated to the Bam HI treated pBinHyg binary vector (Charng et al., 1995), resulting in two plasmids designated pBH40Ts and pBH39Ts.

Plant Transformation

All transformations were performed with tobacco plants containing the Ds::reporter gene construct, and the transgenic tobacco plants were regenerated as described by Charng et al. (1997).

In Vivo and In Vitro Assays for Luciferase Gene Activities

Luciferase enzyme activity was determined as described by Howell et al. (1989) using a Lumat LB 9501 luminometer from Berthold, München, Germany.

For in vivo assaying, the plant material was sprayed with 0.15 mg/l of luciferin aqueous solution, placed in a dark room and then measured by the luminometer immediately. The luminometer consisted of an intensified CCD camera (Hamamatsu, Japan), with a Nikon 35 mm lens, connected to a personal computer. The live plant material image and the luminescent image were taken separately, and the latter revealed callus with luciferase activity.

Genomic DNA Isolation

Genomic DNA was isolated from transformed plants with the use of a kit (BIOL01, Vista, AC). Half gram calli were collected, frozen with liquid nitrogen in a mortar, and ground with a pestle. The nuclei were collected and lysed by protease treatment according to the manufacturer’s instructions. Genomic DNA was precipitated by adding ethanol and dissolved in 100 µl TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0).

Analysis of Ds Excision Events by Polymerase Chain Reaction

For the analysis of Ds transposition from the Ds::LUC construct in transgenic tobacco plants, three synthetic oligonucleotide primers were used: ACP (complementary to the Ac sequence from nucleotide 480-462, 5'-
CTGGGAGACAGGGAGAGTC-3'), primer LUC (complementary to the luciferase coding sequence from position 577 to 556 as numbered by De Wet et al. (1987), 5'-CGGGAGGTAGATGAGATGTGAC-3'), and primer 35S (identical to the CaMV 35S RNA promoter sequence, 5'-TCTTTCGCAAGACCCCTTCC-3'). Each reaction mixture contained ca. 0.1 µg template DNA, 0.25 µg of each primer, 0.2 mM deoxynucleoside triphosphate, 1 U of Taq DNA polymerase, 1.5 mM MgCl2 and 10x buffer. The amplification protocol comprised 40 cycles of 1 min at 94°C, 30 s at 50°C, and 30 s at 72°C.

Results

Construction of the PR-1a::SVTPase Chimeric Gene and its Introduction into Tobacco

In order to improve the usage of the inducible transposon system, we had previously transformed a two-component system into *Nicotiana tabacum* cvs. Samsun nn; i.e. the first component for detection of *Ds* excision efficiency and the second component for providing the TPase source (Figure 1). In this study, various second components were constructed based on the previous two-component system (Charng et al., 1997).

For the first component, a *Ds* element was inserted between the CaMV 35S promoter and the coding region of the luciferase (LUC) reporter gene from firefly (*Ds::LUC*; Figure 1A). This leads to the inactivation of the LUC gene, which is restored upon removal of the *Ds* element from the chimeric gene by transposon excision. This gene construct has been inserted in the binary vector Bin19 (Bevan, 1984) and used to transform tobacco plants. Transformants were selected by maintaining the leaf disks and the regenerating plantlets on kanamycin-containing medium.

For the second component, as a source for the NLS containing TPase, plasmid pBinHygTs was used (Charng et al., 1995). This plasmid contains the PR-1a promoter fusion with a full length cDNA clone of the Ac TPase transcript and was used to provide the native TPase source in this report (as a control). Based on this construct, we then generated two additional second components (Figure 1B). First, the full length *Ac* TPase gene was fused to the typical NLS of SV40 (referred as PR-1a::SV40TPase). The second construct, designated PR-1a::SV39TPase, was a deleted derivative of the PR-1a::SV40TPase. It lacks the first NLS and retains only the second NLS of the putative TPase, but harbors the prototype NLS of SV40.

The kanamycin-resistant progeny of self-pollinated transformants containing the first component (*Ds::LUC* construct) were transformed with the second component, and the transformants were selected on hygromycin B- and kanamycin-containing medium. All transformed tobacco plants contained the same *Ds::LUC* construct and a second component, according to the TPase source. We termed the transformed tobacco plants N- (containing the native TPase), 39- (containing the SV39TPase construct), or 40- (containing the SV40TPase construct). The *Ds* excision events were monitored by analyzing the transformants for reporter gene activities.

Spontaneous Transposition of *Ds* Element in Shoot Derived from Primary Transformed Calli

To determine whether *Ds* undergoes spontaneous transposition, we assayed luciferase activity in the primary regenerated shoots of tobacco transformants. Twenty independent transformed lines for each construct were extracted for luciferase activity assay. For the transformed lines harboring the native TPase source (PR-1a::TPase), 5 out of 20 independent transformed tobacco shoots (N3, N10, N12, N16 and N20) exhibited luciferase activity. For the transformed plants containing PR-1a::SV39TPase

![Figure 1. Schematic representation of two-component system used to access the activity of the Ac transposase under the influence of NLS of SV40.](image-url)
construct, 8 out of 20 independent transformed shoots (39-2, 39-4, 39-5, 39-7, 39-10, 39-11, 39-15 and 39-17) exhibited luciferase activity, and of the transformed shoots containing PR-1a::SV40TPase construct, 11 out of 20 independent transformed shoots (40-1, 40-4, 40-6, 40-9, 40-11, 40-12, 40-15, 40-16, 40-18 and 40-19) exhibited luciferase activity (Table 1). These results indicate that Ds can be triggered spontaneously, and the transposition efficiencies were 25%, 40% and 55% for PR-1a::TPase, PR-1a::SV39TPase and PR-1a::SV40TPase construct, respectively.

To verify that the observed reporter gene activities were due to excision of the Ds element, we analyzed genomic DNA from plants by multiplex PCR with primers LUC (complementary to the luciferase coding sequence), ACP (complementary to the Ac sequence), and 35S (identical to a region of the 35S promoter) (Figure 2A). With primers 35S and ACP, a 580 bp PCR product was obtained with DNA from LUC– tissue of either transformed tobacco plants N1, 39-1 or 40-2 (Figure 2B; lane 1, 3 and 5). In the presence of all three primers, no 670 bp product was generated from DNA of those transformants with 35S and LUC. The distance between these two primers is about 4 kb in the intact Ds::reporter gene construct. Together, these results indicate that Ds element had not undergone transposition in LUC– tissue. In contrast, genomic DNA from LUC+ tissue of either transformed tobacco plants N16, 39-10 or 40-1 yielded PCR products of 580 and 670 bp (Figure 2B), indicating that the LUC+ tissue contained both cells in which Ds had undergone transposition and cells in which it had not.

Table 1. Spontaneous transposition of the Ds element in primary transformed tobacco plants. Luciferase activity was measured in shoots of transformed tobacco plants harboring the native transposase (TPase), SV39TPase (39TPase) or SV40TPase (40TPase) construct. Activity is expressed in relative light units (emission was measured for 2 s) per microgram of total protein; values of <50 RLU mg⁻¹ produced no typical luciferase-luciferin activities corresponding to the absence of luciferase activity, referred to as LUC-.

<table>
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<th>Tobacco/(TPase)</th>
<th>LUC RLU</th>
<th>Tobacco/(39TPase)</th>
<th>LUC RLU</th>
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Figure 2. PCR analysis of \( Ds \) excision from the \( Ds::LUC \) chimeric gene. A, Structure of the \( Ds::LUC \) chimeric gene and the location of primers (shown as solid triangles) used for PCR analysis. The sizes of expected PCR products (580 and 670 bp before and after excision of \( Ds \), respectively) are indicated. B, Ethidium bromide-stained agarose gel on which PCR products were separated. PCR was performed with genomic DNA from the indicated transformants. Lane M, 100 bp DNA ladder.

For in vivo assay of luciferase activities, \( Ds \) excision events are revealed as light images produced by the tissue. We observed that reporter gene activities were always restricted to the calli (Figure 3). This observation indicated that the \( Ds \) excision events were triggered mainly during the development of calli, mostly regenerated from the edge of excised leaf discs (Charng et al., 1997). Furthermore, when the light intensities of LUC+ leaf discs for each construct were used as a record, a similar \( Ds \) excision efficiency was observed for each NLS-TPase construct (see below).

For in vitro assay, the regeneration calli for each transformed line were collected and extracted for luciferase activity assay. Figure 4 shows the mean luciferase activity yielded by each NLS-TPase transformed line with various SA-treatments. With in vitro assay, light is emitted as a peak because the luciferin-luciferase reaction is rapidly feedback-inhibited by a reaction product. All un-induced leaf discs yielded no typical luciferase activity kinetics but background values (RLU<50). These findings suggest that
no spontaneous transposition event occurred. When the leaf discs of these transformed lines were induced with 1 mM SA, the transformed lines harboring the native PR-1a::TPase yielded typical peaks of luciferase light emission, but the LUC activities were low. For the same SA-treatment, transformed plants containing PR-1a::SV40TPase or PR-1a::SV39TPase construct yielded about twofold as much as the transformed plants containing the native PR-1a::TPase construct. On the other hand, when the leaf discs of these transformed lines were induced with 5 mM SA, the transformed lines harboring the native PR-1a::TPase yielded a threefold increase in LUC activities over 1 mM SA-treated tobacco. Furthermore, the 5 mM SA-treated transformed tobacco containing PR-1a::SV40TPase and PR-1a::SV39TPase construct yielded about fourfold and threefold, respectively, what the tobacco containing native PR-1a::TPase construct with the same treatment yielded. Taken together, these results show that the transposition efficiency of Ds was induced by SA in a dose-dependent manner. Furthermore, the NLS-containing TPase can trigger higher Ds transposition more efficiently than the native TPase.

DNA Blot Analysis of Somatic Excisions Arising from the NLS TPase Fusions

To determine whether the somatic excisions triggered by TPase-derived constructs were bona fide transposition events, DNA was extracted from SA-induced transgenic tobacco plants with two components, which yielded various Ds excision efficiencies after 5 mM SA treatment as described above. As probe, the 1.2 kb Bam HI/Eco RV fragment comprising the LUC reporter gene was used. Bands of 4.5 kb (if Ds is not excised) and 1.5 kb (if Ds is transposed from its donor site) can be expected. As shown in Figure 5a, plants with the highest luciferase activity (40-3) showed only the 1.5 kb band but no detectable 4.5 kb band. This indicates an early excision event during regeneration in this plant. In tobacco plants, which contained both the 4.5 kb band and the 1.5 kb fragments, transposition of Ds was incomplete or occurred at a later stage of development. In these plants the luciferase activities are proportional to the relative intensity of the 1.5 kb band (Figure 5a). In plant N1, only a 4.5 kb band was observed. This plant showed no enzyme activity and, as such, suggested an inactive transposon. The same filter was also probed with a 2.9 kb Ds fragment. Bands corresponding to the non-transposed donor site can hybridize to both the LUC probe and the Ds probe, yielding a band of 4.5 kb. Additional bands of various sizes (all larger than 2.9 kb), which hybridized to Ds probe but not to LUC probe, indicated the reinsertion of Ds. As shown in Figure 5b, plant 39-6 yielded three and plant 40-3 yielded four Ds-specific bands.

Discussion

Transposable elements have proven to be a powerful genetic tool for functional genomics studies. Several strategies have been applied to maximize the transposition efficiency the Ac/Ds system in heterologous plants: (1) fusion of a constitutive or inducible promoter to control the expression level of TPase (Scofield et al., 1992; Charng et al., 1995), and (2) application of a demethylating agent, 5AzaC, to regulate the transposition mechanism (Scortecci et al., 1997; Charng et al., 2000). Interestingly, by fusing the TPase with the CaMV 35S promoter, Scofield et al. (1992) found that, in tobacco, the accumulation of high levels of the Ac TPase may inhibit subsequent transposon
excision. This compels us to develop another strategy to improve transposition efficiency. The native Ac TPase has three NLSs near its amino-terminal end, NLS (44-62), NLS (159-178) and NLS (174-206). However, all three were determined to be “weak” NLSs or NLS-like signals (Wang, 1998) though each is sufficient to direct GUS to the nucleus (Boehm et al., 1995). Here, we fused a classical nuclear localization signal (NLS) of SV40 with the TPase gene and tried to increase the nuclear import efficiency of TPase and consequently raise its transposition frequency. Indeed, after SA induction, the fusion TPase harboring NLS triggered a transposition efficiency that was fourfold the native TPase triggered in tobacco.

A curious aspect of the Ac TPase has been reported. A truncated TPase lacking 102 amino acids from the amino-terminus is still functional in transgenic tobacco and Arabidopsis (Li and Starlinger, 1990; Grevelding et al., 1992). In this work, in addition to the full length NLS-TPase construct, we have constructed a similarly truncated NLS-fused TPase (SV39TPase). Our results indicated that when these two kinds of NLS-fused TPase genes were expressed under the control of the PR-1a promoter, the tobacco plants harboring SV40TPase construct always yielded higher transposition efficiency than the plants harboring SV39TPase did. Previously, Heinlein et al. (1994) suggested that a combination of several NLSs of the Ac TPase is required for efficient nuclear transport. On the other hand, these authors also suggested that Ac TPase that forms large aggregates in nuclear and the N-terminally truncated TPase derivative is inefficiently transported into the nucleus and aggregates predominantly in the cytoplasm (Heinlein et al., 1994). The fusion of NLS of SV40 may interfere with the formation of aggregates and leave more free and active TPase to perform the Ds excision. Alternatively, owing a higher number of NLSs, nuclear uptake of SV40TPase may proceed more quickly and lead to higher transposition frequency.

The Ac/Ds transponson system has been widely used to create knockout mutants in many heterologous plants. In order to maximize the Ac transposition efficiencies in many species, we have developed several inducible transposon systems (Charng et al., 1995; 1997; 2000). These systems have demonstrated functionality in tobacco, tomato and rice plants. Here, a new strategy, of fusing the TPase with the truncated TPase derivative is inefficiently transported into the nucleus and aggregates predominantly in the cytoplasm (Heinlein et al., 1994). The fusion of NLS of SV40 may interfere with the formation of aggregates and leave more free and active TPase to perform the Ds excision. Alternatively, owing a higher number of NLSs, nuclear uptake of SV40TPase may proceed more quickly and lead to higher transposition frequency.

The Ac/Ds transposon system has been widely used to create knockout mutants in many heterologous plants. In order to maximize the Ac transposition efficiencies in many species, we have developed several inducible transposon systems (Charng et al., 1995; 1997; 2000). These systems have demonstrated functionality in tobacco, tomato and rice plants. Here, a new strategy, of fusing the TPase with the putative NLS, was introduced for expanding the usage of the inducible transposon systems. All these efforts will allow us to develop more efficient transposon systems for future plant functional genomic studies.

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Literature Cited


A c / D s 轉位子之轉位效率

轉位酶與典型之核定位信號融合後增加 Ac 轉位子之轉位效率

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轉位酶與典型之核定位信號融合後增加 Ac 轉位子之轉位效率

關鍵詞：Ac 轉位酶；「可誘導轉位子」；冷光報導基因；核定位信號。