Journal of Plant Sciences
2016; 4(5): 106-112
http://www.sciencepublishinggroup.com/j/jps
doi: 10.11648/j.jps.20160405.13
ISSN: 2331-0723 (Print); ISSN: 2331-0731 (Online)

Evaluation of the Diphtheria Toxin A Subunit Gene (*DT-A*) as a Non-conditional Negative Selectable Marker in Tobacco and Rice

Pachamuthu Kannan*, Bharat Bhusan Majhi, Karuppannan Veluthambi

Department of Plant Biotechnology, School of Biotechnology, Madurai Kamaraj University, Madurai, Tamil Nadu, India

Email address:

planttechkanna@gmail.com (P. Kannan)

*Corresponding author

To cite this article:

Pachamuthu Kannan, Bharat Bhusan Majhi, Karuppannan Veluthambi. Evaluation of the Diphtheria Toxin A Subunit Gene (*DT-A*) as a Non-conditional Negative Selectable Marker in Tobacco and Rice. *Journal of Plant Sciences*. Vol. 4, No. 5, 2016, pp. 106-112.
doi: 10.11648/j.jps.20160405.13

Received: July 21, 2016; **Accepted:** August 1, 2016; **Published:** August 29, 2016

Abstract: The diphtheria toxin A subunit gene (*DT-A*) from *Corynebacterium diphtheriae* inhibits protein synthesis in eukaryotes. In this study, toxicity of the *DT-A* gene was evaluated by a transgenic approach in tobacco and rice. The *DT-A* gene was cloned under transcriptional control of the CaMV 35S promoter and transformed into tobacco. Similarly, CaMV 35S and the maize *Ubi1* promoter-driven *DT-A* gene constructs were transformed into rice. The deployment of the *DT-A* gene in both tobacco and rice drastically reduced the recovery of transgenic plants in comparison to pCAMBIA1301 (without *DT-A*). Southern blot analyses of the transgenic plants were done using the *hph*- and *DT-A* gene-specific probes to check the presence of the *hph* and *DT-A* genes. All the tobacco and rice transgenic plants showed hybridization to junction fragments upon using the *hph* gene probe. Southern blotting with the *DT-A* probe revealed that all the transgenic plants either did not have the *DT-A* gene or harboured truncated *DT-A* gene in the integrated T-DNAs. None of the transgenic plants carried the complete *DT-A* gene. The results showed that the *DT-A* gene can be used as a good non-conditional negative selectable marker in both tobacco and rice. Both CaMV 35S promoter- and *Ubi1* promoter-driven *DT-A* genes were effective as non-conditional negative selectable markers in rice.

Keywords: *Agrobacterium tumefaciens*, Diphtheria Toxin, Negative Selectable Marker, Rice, Tobacco

1. Introduction

Expression of the negative selectable marker (NSM) genes in transgenic plants causes immediate or conditional cell death by interfering with normal growth and development. It allows selection of cells, which lack the NSM gene [1]. Based on the mode of action, NSMs are classified as non-conditional (substrate-independent) NSM and conditional (substrate-dependent) NSM. Expression of the non-conditional NSM genes leads to immediate cell death after transformation. It does not require any other substrate for its action. Examples of non-conditional NSM are *Corynebacterium diphtheriae* toxin A-chain fragment (*DT-A*) [2], *Pseudomonas aeruginosa* exotoxin A [3], *barnase* from *Bacillus amyloliquefaciens* [4], anti-*nptII* gene [5] and *DIANTHIN* gene from *Dianthus caryophyllus* [6, 7]. In contrast, expression of conditional NSMs does not directly cause lethality to the cells. Instead, it converts a non-toxic substrate to a cytotoxic compound. Cytosine deaminase (*codA)*, indoleacetamide hydrolase (*tms2*), dehalogenase (*dhlA*), cytochrome P450 monooxygenase (*P450*), alcohol dehydrogenase (*cue*), D-amino acid oxidase (*dao1*) and herpes simplex virus thymidine kinase (*HSVtk*) [7, 8] are the examples of conditional NSM. For example, *codA* conditional NSM converts non-toxic 5-fluorocytosine (5-FC) into toxic 5-fluorouracil (5-FU), and has proved to be an efficient conditional NSM in both tobacco and rice [8, 9, 10].

NSMs have many applications in plant biology research. The *DT-A* and *codA* genes have been used in homologous recombination based 'gene targeting' experiments to eliminate the random T-DNA integration events [10, 11, 12], which frequently occur. Selectable marker-free transgenic rice line

was developed by using the *Mungbean yellow mosaic virus* (MYMV) transcriptional activator protein gene (*TrAP*) as a non-conditional NSM [13]. Male sterile transgenic plants were developed using *barnase* as an NSM [14] and also NSMs have been used to eliminate vector backbone sequence in *Agrobacterium*-mediated plant transformation [15].

The diphtheria toxin A-chain fragment gene (*DT-A*) from *Corynebacterium diphtheriae* causes cell death by NAD^+-dependent ADP-ribosylation of elongation factor 2 and inhibits protein synthesis [12, 16]. Diphtheria toxin B chain fragment is required for recognition of cell membrane receptors and is responsible for cell to cell movement of the DT-A protein [12]. Since the *DT-A* gene does not encode the B chain fragment, the cell to cell movement of the DT-A toxin is restricted. The DT-A protein exhibited a high level of toxicity to tobacco [2] and rice cells [17].

In this study, we evaluated the usefulness of the *DT-A* gene as a non-conditional NSM in tobacco and rice. First, a CaMV 35S promoter-driven *DT-A* transgenic tobacco plants were developed. Next, CaMV 35S promoter- and *Ubi1* promoter-driven *DT-A* transgenic rice plants were generated. Transformation of plants with the NSM gene should reduce the recovery of transgenic plants and the recovered transgenic plants are expected to show truncated T-DNAs either without NSM or with truncated NSM gene [8, 11, 17]. This approach was used to study the toxicity of the *DT-A* gene in both tobacco and rice. The results showed that CaMV 35S promoter-driven *DT-A* served as a good non-conditional NSM in tobacco. Both CaMV 35S promoter- and *Ubi1* promoter-driven *DT-A* genes served as good non-conditional NSMs in rice.

2. Materials and Methods

2.1. Construction of Binary Vectors

The binary vector pCam-P35S-*DT-A*-35S 3' was constructed as follows: The *DT-A* gene (585-bp) was amplified from the plasmid pLMY101 [18] as per the following PCR conditions: Initial denaturation at 94°C for 2 min, 30 cycles at 94°C for 40 s, at 58°C for 40 s and at 72°C for 45 s and a final extension at 72°C for 7 min with primers 5'- TACC*ATGGATCCT*GATGATGTTGTTGATTCTTC-3' (the start codon and the codons for two modified amino acids are shown in italic) and 5'-*TCACAAAGA*TCGCCTGACACGATTTCCTGC-3' (the stop codon and the codons of the two introduced amino acids are shown in italic). The amplified fragment was cloned in pGEM-T (Promega, Madison, USA). The primers were designed to introduce the start (ATG) and stop (TGA) codons in the *DT-A* gene. The first two aminoacids of the native *DT-A* gene, glycine (GGC) and alanine (GCT), were modified to aspartate (GAT) and proline (CCT) and two additional aminoacids, serine (TCT) and leucine (TTG), were introduced before the stop codon, as reported previously [2, 19]. The *DT-A* ORF was checked and verified by sequencing. The *DT-A* gene was cloned in the MCS of pJIC35S [20] to yield the P35S-*DT-A*-35S 3' cassette. The 1.3-kb P35S-*DT-A*-35S 3' *Eco*RV fragment was cloned in the *Sma*I site of pCAMBIA1301 to yield pCam-P35S-*DT-A*-35S 3'. The binary vector was mobilized into *Agrobacterium tumefaciens* LBA4404 (pSB1) [21].

The binary vector pCam-P*Ubi1-DT-A*-35S 3' was constructed as follows: A 0.9-kb *Eco*RV/*Sac*II fragment containing the *DT-A* coding sequence with the 35S 3' was cloned in the respective sites of pBS(II)KS^+ (Stratagene, West Cedar, USA). The 0.9-kb *DT-A* gene with 35S 3' was excised as an *Eco*RV/*Sac*I fragment and cloned downstream of the *Ubi1* promoter. The 2.9-kb P*Ubi1-DT-A*-35S 3' cassette was taken as a *Stu*I/*Eco*RV fragment and subcloned in the *Sma*I site of pUC18. The 2.9-kb P*Ubi1-DT-A*-35S 3' cassette was excised as a *Hin*dIII/*Kpn*I fragment and cloned in the corresponding sites of pCAMBIA1301 to yield the binary plasmid pCam-P*Ubi1-DT-A*-35S 3', which was mobilized into the *A. tumefaciens* strain LBA4404 (pSB1) by triparental mating.

2.2. Transformation of Tobacco and Rice

The *A. tumefaciens* LBA4404 (pSB1, pCamP35S-*DT-A*-35S 3') strain was used for tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) transformation. Tobacco leaf discs (8 mm diameter) were infected with the *Agrobacterium* strain as described by Sunilkumar *et al*. (1999) [22]. *Agrobacterium*-infected tobacco leaf discs were maintained on the shoot-induction medium [Murashige and Skoog (MS) salts [23], B5 vitamins, 0.5 µM NAA, 4 µM BAP, 3% (*w*/*v*) sucrose, 0.8% (*w*/*v*) agar, pH 5.7] which contained 50 mg L^{-1} hygromycin (Hyg) and 250 mg L^{-1} cefotaxime. The Hyg^R transgenic shoots were kept for root induction on the BGS medium (MS salts, 1 mg L^{-1} folic acid, 100 mg L^{-1} myoinositol, 0.4 mg L^{-1} thiamine, 0.057 µM indole-3-acetic acid, 0.14 µM kinetin, 3% [*w*/*v*] sucrose, 0.9% [*w*/*v*] agar, pH 5.7) supplemented with 250 mg L^{-1} cefotaxime and 50 mg L^{-1} hygromycin. Scutellum-derived rice (*Oryza sativa* L. subsp. *indica* cv. Pusa Basmati 1) calli were used for *Agrobacterium*-infection (pCam-P35S-*DT-A*-35S 3' or pCam-P*Ubi1-DT-A*-35S 3'). Callus induction, *Agrobacterium*-mediated transformation, selection and regeneration were done as described by Sridevi *et al*. (2003) [24]. GUS histochemical staining was done as described earlier by Sunilkumar *et al*. (1999) [22].

2.3. Southern Blot Analysis

Total DNA was extracted from tobacco and rice leaves [25]. DNA was estimated using the Hoechst dye 33258 in the DyNa Quant 200 fluorometer (Hoefer Scientific Instruments, San Francisco, USA). For Southern blot analysis, 2.5 µg of rice DNA and 10 µg of tobacco DNA was digested with appropriate restriction enzymes and electrophoresed in 0.8% agarose gels in 1x Tris-borate-EDTA (TBE) buffer. The DNA was transferred onto Zeta probe nylon membrane (Bio-Rad, Hercules, USA). The [α-^{32}P]dCTP-labelled *hph* and *DT-A* gene fragments were used as probes.

3. Results and Discussion

3.1. Transformation of Tobacco with the DT-A Gene Under the Transcriptional Control of CaMV 35S Promoter

Tobacco leaf discs were used for transformation with the binary vector pCAMBIA1301 (without the *DT-A* gene) and pCam-P35S-*DT-A*-35S 3'. pCAMBIA1301 yielded 100% (60 out of 60) transformation of tobacco leaf discs. However, pCam-P35S-*DT-A*-35S 3' yielded only four Hyg^R plants out of 100 leaf discs (Table 1). Four Hyg^R tobacco plants obtained with pCam-P35S-*DT-A*-35S 3' were subjected to Southern blot analysis to study the T-DNA integration. Plant DNA was digested with *Eco*RI. The blot was first probed with the [α-^{32}P]dCTP labelled-*hph* gene. All four Hyg^R tobacco plants displayed hybridization of junction fragments longer than 2.1 kb, which confirmed the integration of T-DNA (Fig. 1a, b). If the complete *DT-A* gene is integrated in the transgenic tobacco plants, an internal T-DNA fragment of 0.6-kb is expected to hybridize upon *Eco*RI + *Hin*dIII digestion of plant DNA (Fig. 1a). Control and transgenic tobacco DNA was digested with *Eco*RI + *Hin*dIII and the blot was probed with the *DT-A* gene. The Southern blot results showed that transgenic tobacco plant 2 and 4 did not harbour the *DT-A* gene. Transgenic tobacco plant 1 showed hybridization close to the 0.6-kb position (Fig. 1c), but the PCR analysis with the *DT-A* gene-specific primers did not amplify the 0.6-kb *DT-A* gene (Fig. 1d). The result showed that the *DT-A* gene in tobacco plant 1 is truncated and did not have an annealing site of one of the two primers. Transgenic tobacco plant 3 showed hybridization to a 2.8 kb junction fragment which was longer than the expected 0.6-kb internal T-DNA fragment (Fig. 1c). The results indicated that none of the four transgenic tobacco plants carried the complete *DT-A* gene. Thus, *DT-A* can be effectively used as a non-conditional NSM in tobacco.

Table 1. *Comparison of tobacco and rice transformation with* A. tumefaciens *LBA4404 (pSB1) which harboured binary plasmids without the* DT-A *gene (pCAMBIA1301) and with the* DT-A *gene (pCam-P35S*-DT-A-*35S 3' and pCam-P*Ubi1-DT-A-*35S 3').*

S. No	Binary plasmid	Host plant	No. of tobacco leaf discs/rice calli used for transformation	No. of leaf discs/rice calli which yielded Hyg^R plants	Transformation efficiency
1	pCAMBIA1301	Tobacco	60	60	100%
2	pCam-P35S-*DT-A*-35S 3'	Tobacco	100	4	4%
3	pCAMBIA1301	Rice	200	28	14%
4	pCam-P35S-*DT-A*-35S 3'	Rice	280	3	1.1%
5	pCAMBIA1301	Rice	80	11	13.7%
6	pCam-P*Ubi1-DT-A*-35S 3'	Rice	150	1	0.6%

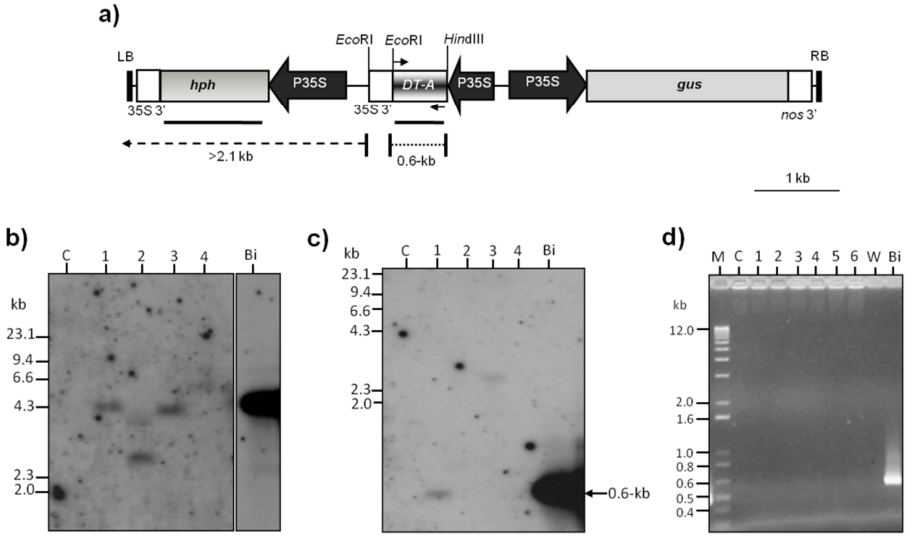

Figure 1. *Analysis of tobacco plants transformed with pCam-P35S-*DT-A-*35S 3'. a) T-DNA of the binary plasmid pCam-P35S-*DT-A-*35S 3' which was used for both tobacco and rice transformation. The left border (LB) junction fragment marked with a broken line with an arrow would hybridize to the* hph *probe (marked with a bold line). The 0.6-kb* DT-A *gene (marked with a bold line), flanked by* Eco*RI and* Hind*III, will hybridize to the* DT-A *gene probe. P35S,* Cauliflower mosaic virus *35S promoter; 35S 3',* Cauliflower mosaic virus *35S polyA signal; hph, hygromycin phosphotransferase selectable marker gene; gus, β-glucuronidase gene; nos 3', nopaline synthase gene polyA signal. Positions of the primers used for amplification of the* DT-A *gene are marked in arrows. b) Southern blot analysis of pCam-P35S-*DT-A-*35S 3'-transformed tobacco plants using the* hph *gene probe. Control, untransformed (C) and transgenic tobacco plant (1 to 4) DNA was digested with* Eco*RI and the blot was probed with the [α-^{32}P]dCTP-labelled* hph *probe. The binary plasmid pCam-P35S-*DT-A-*35S 3' (lane Bi, 50 pg) digested with* Eco*RI was used as a positive control. c) Southern blot analysis of transgenic tobacco plants transformed with pCam-P35S-*DT-A-*35S 3' using the* DT-A *gene probe. Control, untransformed (C) and transgenic tobacco plant (1 to 4) DNA was digested with* Eco*RI +* Hind*III and the blot was probed with the [α-^{32}P]dCTP-labelled* DT-A *gene. Binary plasmid pCam-P35S-*DT-A-*35S 3' digested with* Eco*RI +* Hind*III was used as a positive control (Bi). Sizes of the λ/*Hind*III fragments are positioned on the left. d) PCR analysis of pCam-P35S-*DT-A-*35S 3'-transformed tobacco plants using the* DT-A-*gene specific primers. Lanes 1 to 4 are Southern positive plants transformed with pCam-P35S-*DT-A-*35S 3'. Lanes 5 and 6 are pCAMBIA1301 binary plasmid transformed plants used as a negative control. C, Non-transgenic, control tobacco plant DNA; Bi, 50 pg of binary plasmid (pCam-P35S-*DT-A-*35S 3') used as a positive control. W, water control; M, 1 kb$^+$ DNA marker.*

3.2. Transformation of Rice with the DT-A Under the Transcriptional Control of CaMV 35S and the Ubi1 Promoters

To evaluate the toxicity of the *DT-A* gene in rice, the binary plasmids pCam-P35S-*DT-A*-35S 3' (the *DT-A* gene under CaMV 35S promoter) and pCam-P*Ubi1-DT-A*-35S 3' (the *DT-A* gene under maize *ubiquitin* promoter) were constructed and mobilized into *A. tumefaciens* LBA4404 (pSB1). First, pCam-P35S-*DT-A*-35S 3' was used for rice transformation along with pCAMBIA1301 (without *DT-A* gene) as a control. *Agrobacterium*-mediated transformation with pCam-P35S-*DT-A*-35S 3' yielded three Hyg^R rice plants out of 280 calli (1.1% transformation efficiency) but transformation with the control binary vector pCAMBIA1301 yielded 28 plants (out of 200 calli) with 14% transformation frequency (Table 1). Upon Southern blot analysis with the *hph* gene, all three *Eco*RI digested pCam-P35S-*DT-A*-35S 3'-transformed rice plants showed junction fragments longer than 2.1 kb (Fig. 2a). Presence of the complete *DT-A* gene in the T-DNA of the transgenic rice plants was studied by internal T-DNA fragment analysis with the *DT-A* probe. Control and transgenic rice plant DNA was digested with *Eco*RI + *Hin*dIII and the blot was probed with the *DT-A* gene. Transgenic plants 2 and 3 did not display hybridization of the expected 0.6-kb internal T-DNA fragment (Fig. 2b). Plant 1 did not hybridize to the expected 0.6-kb fragment but hybridized to a 2.2 kb fragment, indicating the presence of an incomplete *DT-A* gene which formed a junction fragment (Fig. 2b). The Southern blotting result conclusively showed that none of the three transgenic rice plants harboured the complete *DT-A* gene. Toxicity of the *DT-A* gene is responsible for eliminating the events which contained complete T-DNA with an intact copy of the *DT-A* gene.

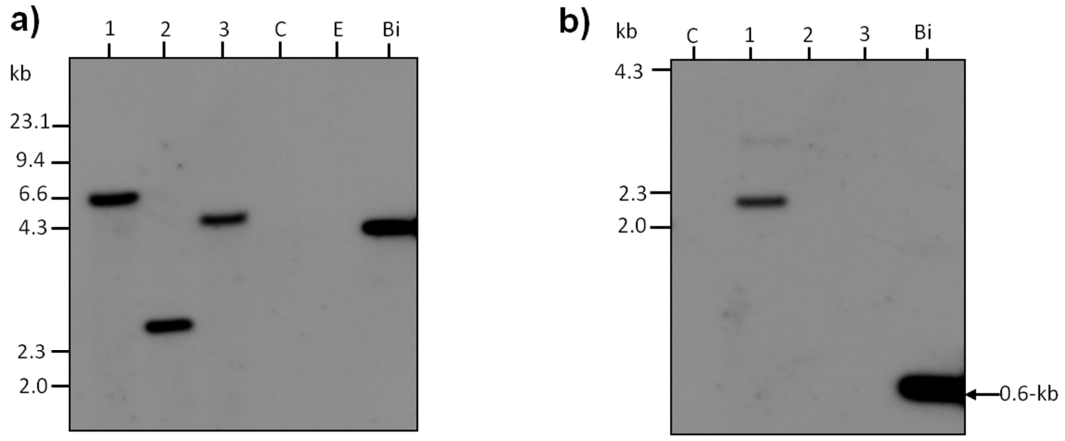

Figure 2. *Southern blot analysis of pCam-P35S-*DT-A-*35S 3'-transformed rice plants. a) Analysis of left border junction fragments with the [α-^{32}P]dCTP-labelled* hph *probe. Total DNA (2.5 µg) of control, untransformed (C) and three transformed Hyg^R transgenic rice plants (1 to 3) was digested with* Eco*RI and the blot was probed with the* hph *gene. Bi, Binary plasmid pCam-P35S-*DT-A-*35S 3' digested with* Eco*RI (50 pg) was used as a positive control. E, empty lane. b) Analysis of integration of the complete* DT-A *gene in the transgenic rice plants with the [α-^{32}P]dCTP-labelled* DT-A *gene probe. DNA of control (C) and transgenic rice plants (1 to 3) was digested with* Eco*RI +* Hind*III and the blot was probed with the [α-^{32}P]dCTP-labelled* DT-A *gene. The binary plasmid pCam-35S-*DT-A-*35S 3' was digested with* Eco*RI +* Hind*III and used as a positive control (lane Bi, 50 pg). Sizes of the λ/*Hind*III fragments are positioned on the left.*

The CaMV 35S promoter is most frequently used in plants for expressing transgenes in a constitutive manner [26]. It has been reported that CaMV 35S promoter showed high level transgene expression in dicotyledonous plants compared to monocotyledonous plants [27]. Different promoters are needed for multiple transgene expression in a single transgenic plant in order to avoid homology-based gene silencing [28, 29]. Therefore, the toxicity of the *DT-A* gene in rice was also evaluated in this study under the control of the *Ubi1* promoter. The 21-day-old rice scutellum-derived callus was used for transformation with the binary vectors pCAMBIA1301 (without the *DT-A* gene) and pCam-P*Ubi1-DT-A*-35S 3'. pCAMBIA1301 yielded 11 Hyg^R plants out of 80 calli (13.7%) and pCam-P*Ubi1-DT-A*-35S 3' construct yielded only one Hyg^R plant out of 150 calli (0.6%, Table 1). GUS histochemical analysis was done in control, pCAMBIA1301- and pCam-P*Ubi1-DT-A*-35S 3'- transformed rice plants. The pCam-P*Ubi1-DT-A*-35S 3'-transformed plant did not show GUS activity but the pCAMBIA1301-transformed plant displayed GUS staining (Fig. 3a). This result indicated that the T-DNA integrated in the pCam-P*Ubi1-DT-A*-35S 3'-transformed plant did not have the complete T-DNA.

Southern blot analysis was done to study T-DNA integration in the Hyg^R transgenic rice plants. Total DNA (2.5 µg) from transgenic and control rice plants was digested with *Eco*RI and used for junction fragment analysis. Junction fragments longer than 2.1 kb are expected to hybridize to the *hph* probe (Fig. 3b). One Hyg^R rice plant obtained with pCAMBIA1301 and one Hyg^R rice plant obtained with pCam-P*Ubi1-DT-A*-35S 3' were taken for the analysis. Southern blotting with the *hph* probe displayed hybridization to junction fragments longer than 2.1 kb (Fig. 3c) in pCAMBIA1301- and pCam-P*Ubi1-DT-A*-35S 3'-transformed plants. An internal T-DNA fragment of 0.6-kb was expected to hybridize upon *Pst*I digestion, if the complete *DT-A* gene is

integrated in the transgenic rice plant (Fig. 3b). Hybridization of the 0.6-kb fragment was observed in the positive control lane with the binary vector pCam-P*Ubi1-DT-A*-35S 3', but the pCam-P*Ubi1-DT-A*-35S 3'-transformed Hyg^R transgenic plant did not show the hybridization to the *DT-A* probe (Fig. 3d). The result confirmed that the integrated T-DNA did not harbour the *DT-A* gene. These observations correlate well with the previous reports of rice transformation with P35S-*DT-A*-35S 3' and P*Ubi1-DT-A*-35S 3' [17].

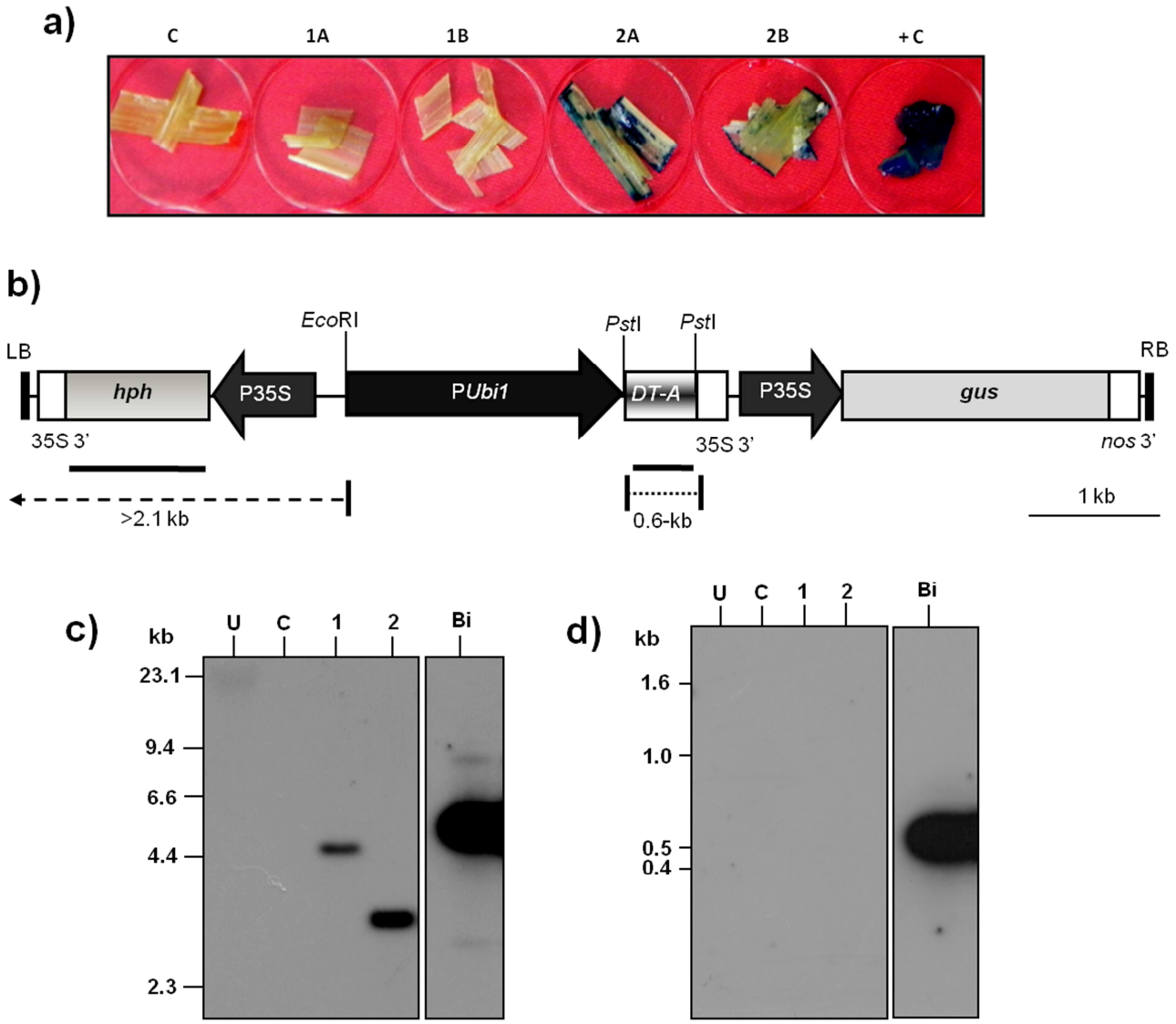

Figure 3. *Characterization of a pCam-P*Ubi1-DT-A-*35S 3'-transformed transgenic rice plant. a) Histochemical analysis of GUS activity in pCam-P*Ubi1-DT-A-*35S 3' and pCAMBIA1301-transformed transgenic rice plants. GUS staining in the leaf. C, Leaf from untransformed control rice plant; +C, previously transformed GUS positive rice calli as a positive control; 1A and 1B, duplicate samples of a pCam-P*Ubi1-DT-A-*35S 3'-transformed plant; 2A and 2B, duplicate samples of a pCAMBIA1301-transformed plant. b) Linear T-DNA map of the pCam-P*Ubi1-DT-A-*35S 3' binary plasmid. P*Ubi1, *maize* ubiquitin promoter. The probes used for Southern blot analyses (*hph *and* DT-A*) are marked in bold lines. The junction fragment and internal T-DNA fragments (>2.1 kb and 0.6-kb) are marked as dotted line with one side arrow and as a continuous line, respectively. c) Southern blot analysis of the transgenic rice plant with the* hph *probe. Left border (LB) junction fragment was analysed with the [α-^{32} P]dCTP-labelled* hph *probe. Total DNA (2.5 µg) from Hyg^R pCAMBIA1301-transformed (lane 1), pCam-P*Ubi1-DT-A-*35S 3'-transformed (lane 2) and control (untransformed) (C) plants was digested with* EcoRI *and the blot was probed with the* hph *gene. d) Southern blot analysis to check the integration of complete* DT-A *gene in the pCam-P*Ubi1-DT-A-*35S 3'-transformed rice plant. Control (C) and transgenic rice plant DNA was digested with* PstI *and the blot was probed with the* DT-A *gene. In c and d, U, undigested plant DNA; C, control, untransformed plant DNA; 1, pCAMBIA1301-transformed plant DNA; 2, pCam-P*Ubi1-DT-A-*35S 3'-transformed plant DNA. Binary plasmid (Bi) (pCam-P*Ubi1-DT-A-*35S 3') digested with* EcoRI *(c) and* PstI *(d) (50 pg) was used as the positive control.*

4. Conclusion

An NSM gene may show toxicity to one particular plant species and may not show toxicity to others. Expression of the *DIANTHIN* gene caused lethality in tobacco cells but not in rice [7]. Similarly, the *ABRIN-A* gene showed toxicity in tobacco but not in rice [8]. In this study, the *DT-A* gene under the control of constitutive promoters was transformed in both tobacco and rice. *Agrobacterium*-mediated transformation with the *DT-A* gene constructs profoundly reduced the

recovery of transgenic plants compared to pCAMBIA1301 (without the *DT-A* gene) transformation (Table 1). All the *DT-A* transformed transgenic tobacco and rice plants displayed truncated T-DNAs with either the truncated *DT-A* gene or without the *DT-A* gene. The results conclusively show that the *DT-A* gene can be effectively used as a non-conditional NSM in both tobacco and rice. Both CaMV 35S promoter- and maize *Ubi1* promoter-driven *DT-A* genes are effective in rice.

References

[1] Koprek, T., McElroy, D., Louwerse, J., Williams-Carrier, R., Lemaux, P. G (1999) Negative selection systems for transgenic barley (*Hordeum vulgare* L.): comparison of bacterial *cod* A-and cytochrome P450 gene-mediated selection. Plant J 19, 719-726.

[2] Czako, M., An, G (1991) Expression of DNA coding for diphtheria toxin chain A is toxic to plant cells. Plant Physiol 95, 687-692.

[3] Koning, A., Jones, A., Fillatti, J. J., Comai, L., Lassner, M. W (1992) Arrest of embryo development in *Brassica napus* mediated by modified *Pseudomonas aeruginosa* exotoxin A. Plant Mol Biol 18, 247-258.

[4] Mariani, C., De Beuckeleer, M., Truettner, J., Leemans, J., Goldberg, R. B (1990) Induction of male sterility in plants by a chimaeric ribonuclease gene. Nature 347, 737-741.

[5] Xiang, C., Guerra, D. J (1993) The anti-*nptII* gene. A potential negative selectable marker for plants. Plant Physiol 102, 287-293.

[6] Stirpe, F., Williams, D. G., Onyon, L. J., Legg, R. F., Stevens, W. A (1981) Dianthins, ribosome-damage proteins with anti-viral properties from *Dianthus caryophyllus* L. (carnation). Biochem. J 195, 399-405.

[7] Shah, J. M., Veluthambi, K (2010) *DIANTHIN*, a negative selection marker in tobacco, is non-toxic in transgenic rice and confers sheath blight resistance. Biol Plant 54, 443-450.

[8] Majhi, B. B., Bhosale, R., Jawkar, S., Veluthambi, K (2014) Evaluation of *codA*, *tms2* and *ABRIN-A* as negative selectable markers in transgenic tobacco and rice. In Vitro Cell Dev Biol-Plant 50, 541-551.

[9] Dai, S., Carcamo, R., Zhang, Z., Chen, S., Beachy, R. N (2001) The bacterial cytosine deaminase gene used as a conditional negative selection marker in transgenic rice plants. Plant Cell Rep 20, 738-743.

[10] Osakabe, K., Nishizawa-Yokoi, A., Ohtsuki, N., Osakabe, Y., Toki, S (2013) A mutated cytosine deaminase gene, *codA* (D314A), as an efficient negative selection marker for gene targeting in rice. Plant Cell Physiol 55, 658-665.

[11] Terada, R., Urawa, H., Inagaki, Y., Tsugane, K., Iida, S (2002) Efficient gene targeting by homologous recombination in rice. Nat Biotechnol 20, 1030-1034.

[12] Iida, S., Terada, R (2005) Modification of endogenous natural genes by gene targeting in rice and other higher plants. Plant Mol Biol 59, 205-219.

[13] RamanaRao, M. V., Veluthambi, K (2010) Selectable marker elimination in the T_0 generation by *Agrobacterium*-mediated co-transformation involving *Mungbean yellow mosaic virus TrAP* as a non-conditional negative selectable marker and *bar* for transient positive selection. Plant cell Rep 29, 473-483.

[14] De Block, M., Debrouwer, D., Moens, T (1997) The development of a nuclear male sterility system in wheat. Expression of the *barnase* gene under the control of tapetum specific promoters. Theor Appl Genet 95, 125-131.

[15] Hanson, B., Engler, D., Moy, Y., Newman, B., Ralston, E., Gutterson, N (1999) A simple method to enrich an *Agrobacterium*-transformed population for plants containing only T-DNA sequences. Plant J 19, 727-734.

[16] Pappenheimer, A. M. J (1977) Diphtheria toxin. Annu Rev Biochem 46, 69–94.

[17] Terada, R., Asao, H., Iida, S (2004) A large-scale *Agrobacterium*-mediated transformation procedure with a strong positive-negative selection for gene targeting in rice (*Oryza sativa* L.). Plant Cell Rep 22, 653-659.

[18] Mattheakis, L. C., Shen, W. H., Collier, R. J (1992) *DPH5*, a methyltransferase gene required for diphthamide biosynthesis in *Saccharomyces cerevisiae*. Mol Cell Biol 12, 4026-4037.

[19] Palmiter, R. D., Behringer, R. R., Quaife, C. J., Maxwell, F., Maxwell, I. H., Brinster, R. L (1987) Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene. Cell 50, 435-443.

[20] Hellens, R. P., Edwards, E. A., Leyland, N. R., Bean, S., Mullineaux, P. M (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. Plant Mol Biol 42, 819-832.

[21] Komari, T., Hiei, Y., Saito, Y., Murai, N., Kumashiro, T (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J 10,165-174.

[22] Sunilkumar, G., Vijayachandra, K., Veluthambi, K (1999) Preincubation of cut tobacco leaf explants promotes *Agrobacterium*-mediated transformation by increasing *vir* gene induction. Plant Sci 141, 51-58.

[23] Murashige, T., Skoog, F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15, 473-497.

[24] Sridevi, G., Sabapathi, N., Meena, P., Nandakumar, R., Samiyappan, R., Muthukrishnan, S., Veluthambi, K (2003) Transgenic *indica* rice variety Pusa Basmati 1 constitutively expressing a rice chitinase gene exhibits enhanced resistance to *Rhizoctonia solani*. J. Plant Biochem Biotechnol 12, 93-101.

[25] Rogers, S. O., Bendich, A. J (1988) Extraction of DNA from plant tissues. In: Gelvin S. B, Schilperoort R. A, Verma D. P. S (eds) Plant molecular biology manual, vol A6. Kluwer, Dordrecht, pp 1-10.

[26] Odell, J. T., Nagy, F., Chua, N. H (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313, 810-812.

[27] Gupta, P., Raghuvanshi, S., Tyagi, A. K (2001) Assessment of the efficiency of various gene promoters via biolistics in leaf and regenerating seed callus of millets, *Eleusine coracana* and *Echinochloa crusgalli*. Plant Biotechnol J 18, 275-282.

[28] Potenza, C., Aleman, L., Sengupta-Gopalan, C (2004) Targeting transgene expression in research, agricultural, and environmental applications: promoters used in plant transformation. In Vitro Cell Dev Biol-Plant 40, 1-22.

[29] Park, S. H., Yi, N., Kim Y. S., Jeong, M. H., Bang, S. W., Choi, Y. D., Kim, J. K (2010) Analysis of five novel putative constitutive gene promoters in transgenic rice plants. J Exp Bot 61, 2459-2467.