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Expression of DNA Coding for Diphtheria Toxin Chain A Is Toxic to Plant Cells[1]

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ABSTRACT

DNA coding for the enzymatically active subunit A of diphtheria toxin was placed under the control of the cauliflower mosaic virus 35S promoter and the *Agrobacterium* left transfer-DNA gene 7 polyadenylation signal. Agrobacteria carrying a binary plant vector with the chimeric diphtheria toxin A gene had very low transforming activity in tobacco (*Nicotiana tabacum* L.), and greatly diminished the recovery of stable transformants when mixed together with agrobacteria which alone transformed plant cells well. The introduction of this chimeric molecule into tobacco cells by electroporation lowered the level of the transient expression of the coelectroporated chloramphenicol acetyltransferase reporter gene indicating that expression of diphtheria toxin chain A in plant cells is toxic. We have developed a binary vector pGA987 which can be used for probing a variety of plant promoters.

A great variety of heterologous genes has been introduced into plants by *Agrobacterium*-mediated or direct gene transfer. Some of the introduced genes have become invaluable tools as selectable markers for plant cell transformation, or as reporter genes in transcriptional or translational fusions for studying spatial and temporal features of gene expression. Other genes alter physiology, *i.e.* confer tolerance to viral infections, insect feeding, or herbicides (11).

As reporter genes, the bacterial *cat*[2] and β-glucuronidase genes have been used most widely (14, 19). Both gene activities can be quantified, and the latter can be detected *in situ*; however, the assays are still tedious and destruct the material. In addition, they are sometimes complicated by endogenous inhibitors or background activity (10, 15, 19). In an attempt to identify a "suicide" gene which would have a visible phenotype, we have studied the effects of the expression of the potent intracellular toxin, DTx, from a chimeric gene construct in plants.

DTx is a member of a group of protein toxins with intracellular targets and inhibits protein synthesis. Relatively few eucaryotic species are susceptible to the toxic action of DTx. However, DTx catalyzes the NAD-dependent ADP-ribosylation to inactivate elongation factor-2 in cell-free extracts of all eucaryotic species tested (28), including wheat (5). Recently a second cytotoxic pathway of DTx has been reported, *i.e.* a cation dependent nuclease activity (8), and the active site is distinct from that for the ADP-ribosylation (6). The structural gene for DTx carried by corynebacteriophages codes for a protein with a leader peptide which is removed during secretion from *Corynebacterium diphtheriae*. The mature toxin is composed of two domains: the NH_2-terminal portion (A chain) which carries the active sites of the toxin and the COOH-terminal portion (B chain) which is required for binding to specific receptors, transport of the toxin into target cells, and is dispensable for intracellular toxicity. The toxin protein is unmasked and activated by reduction of the disulfide linkages and by hydrolysis of a peptide bond located in the loop between the sulfur atoms of the disulfide bridge located in the NH_2-terminal portion of the intact toxin. The two fragments remain firmly held together by weak interactions (27).

Because polypeptide fragments of microbial and plant toxins (including DTx) that are devoid of cell receptor binding and transfer domains are not cytotoxic, it has become attractive to use these polypeptides in yeast (29) and mammalian (21, 26) systems in the assembly of chimeric genes. Here we present evidence that expression of a chimeric DTx-A gene construct in tobacco is toxic. This finding provides a potent new negative selection gene for probing the function of plant promoters.

MATERIALS AND METHODS

Strains and Molecular Cloning

Escherichia coli strain MC1000 (7) was used as a recipient for plasmids constructed by standard procedures. The *Agrobacterium tumefaciens* strain LBA4404 (17) carrying the pAL4404 disarmed helper Ti plasmid in a Ach5 chromosomal background was used for plant transformation. The DNA coding for the DTx fragment A was obtained from pTH1 (21). The binary plant expression vectors pGA642 and pGA643, carrying a polylinker site between the nopaline synthase promoter and CaMV 35S promoter, respectively, and the pTiA6 octopine-type Ti-plasmid T_L-DNA 7 gene terminator were described previously (4). The binary vectors were transferred into *Agrobacterium* by the direct transformation method (2).

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[2] Abbreviations: *cat*, chloramphenicol acetyltransferase gene; DTx, diphtheria toxin; DTx-A, chain A of diphtheria toxin; Ti, tumor-inducing; T_L-DNA, left transfer-DNA; CaMV, cauliflower mosaic virus; kbp, kilobasepairs; *nos*, nopaline synthase gene; CAT, chloramphenicol acetyltransferase enzyme; T_R-DNA, right transfer-DNA.

Plant Transformation

Tobacco, *Nicotiana tabacum* L. cv "Petit Havana" SR1, plants (20) were used for leaf segment transformation (4). Briefly, leaf segments were coincubated with the *Agrobacterium* cells for 2 d, then placed directly on selective shoot induction medium with 75 μg/mL kanamycin monosulfate (Sigma).

Tobacco NT1 suspension (1) was used for cultured cell transformation by cocultivation (4). Four milliliters of a 3-d-old suspension culture was inoculated with 50 μL of *Agrobacterium* suspension which was grown to the optical density of 1.3 at 660 nm. In coinfection experiments 25 μL of each bacterial suspension was combined. The cultures were incubated at 28°C for 2 d. Then 1.5 mL aliquots were plated from an appropriate dilution onto MS agar medium with 0.2 μg/mL 2,4-D and 75 μg/mL kanamycin monosulfate.

Transient Assay of Diphtheria Toxin Constructs

Exponentially growing NT1 cells (1) were subjected to digestion essentially according to Nagata *et al.* (24) in a mixture of 0.5% Cellulase ("Onozuka" RS grade, Yakult Honsha Co., Ltd., Tokyo), 0.5% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Tokyo), and 0.4 M mannitol at pH 5.6 for 1 h with gentle shaking during the first 30 min. Cells were harvested by centrifugation, washed with 0.4 M mannitol, and electroporated with plasmid DNA according to Mitra and An (23). Fifty microliters of plasmid DNA solution containing 10 μg of carrier DNA (from salmon sperm) plus various amounts of test plasmid DNAs in TE buffer (Tris 10 mM; EDTA 1 mM [pH 8]) was mixed with 2×10^6 protoplasts in 1 mL electroporation buffer. Cells were harvested 48 h after electroporation and CAT activity of the crude extracts was measured as described earlier (4) using 60 μg protein for the assay. The experiments were carried out in triplicate.

Containment

All experiments were performed in accordance with the NIH guidelines for research involving recombinant DNA molecules with particular reference to the cloned diphtheria toxin gene, as published in Vol. 49, No. 227 of the *Federal Register*.

RESULTS

Construction of a Chimeric DTx-A Gene for Expression in Plant Cells

The DTx-A coding region was taken from pTH1 which contains a chimeric DTx-A gene for expression in mammalian cells (21). To provide the necessary signals for efficient expression in plants, the 0.6 kilobase pair *Sau*3AI fragment carrying the coding region of the A chain of the DTx-A gene was inserted into the *Bgl*II site of the binary plant transformation vector pGA941 to give pGA953 (Fig. 1). pGA941 was derived from pGA643 (4) by inserting the *Nco*I linker hexamer (CCATGG) onto the polylinker region to provide translation initiation codon for the incoming DTx-A region. Due to the insertion of the linker in two copies there are two ATGs in

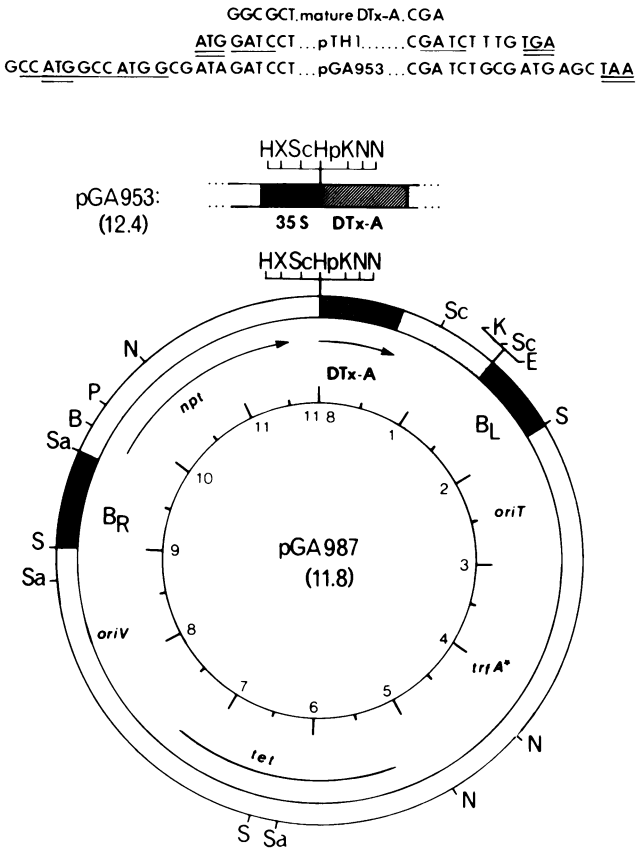

Figure 1. A, Circular map of the binary plant promoter probe vector pGA987. A relevant segment of pGA953 expressing diphtheria toxin chain A is shown above the circular map. pGA953 differs from pGA987 only by the extra 0.4 kbp fragment (black box) containing the CaMV 35S promoter. In pGA953 the coding region for the A chain of diphtheria toxin (shaded box) is under the control of the CaMV 35S promoter and followed by the T_L-DNA gene 7 termination region containing an in frame translation stop codon and polyadenylation signal. Restriction endonuclease sites are indicated on the outer circle. The coordinates on the inner circle indicate the 11.8 kilobase pairs. Nucleotide sequences at the NH_2- and COOH-termini of the DTx-A gene at different stages of manipulation are shown on the top, the translation initiation and stop codons being double underlined. Recognition sites for *Sau*3AI in pTH1 and *Nco*I in pGA953 are underlined. B, *Bgl*II; B_L and B_R, T-DNA border fragments (black boxes); E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; N, *Nco*I; *npt*, chimeric neomycin phosphotransferase selectable plant transformation marker gene; *oriT*; wide host-range plasmid RK2 origin of conjugal transfer; *oriV*, RK2 origin of replication; P, *Pst*I; S, *Sal*I; Sa, *Sac*II; Sc, *Sac*I; *tet*, RK2 tetracycline resistance gene, *trfA**, a segment encoding a replication protein for RK2; X, *Xba*I.

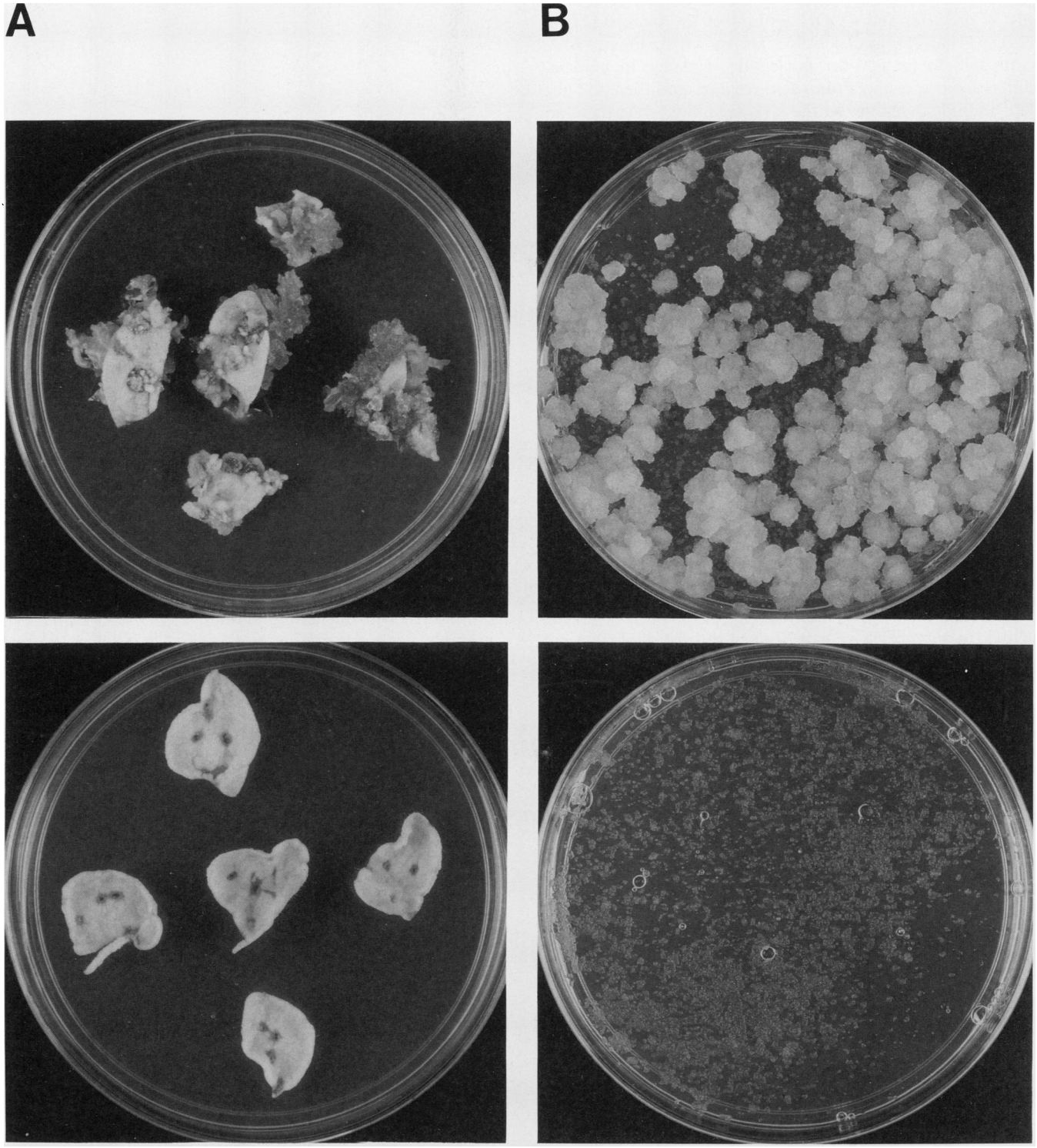

Figure 2. Stable transformation of tobacco leaf segments and cultured cells with DTx-A gene. A, Developing shoots and calli on 75 μg/mL kanamycin containing medium from tobacco leaf sections. Pictures were taken 5 weeks after cocultivation with agrobacteria harboring pGA642 (top) or pGA953 (bottom). B, Emerging transformants on 75 μg/mL kanamycin containing medium from NT1 suspension cells seven weeks after cocultivation with pGA642 (top) or pGA953 (bottom).

frame as verified by sequencing. In pGA953 the DTx-A coding region is under the control of the CaMV 35S promoter and the T_L-DNA gene 7 terminator region. The open reading frame is terminated at a TAA codon randomly occurring in the terminator region. The nucleotide sequences corresponding to the NH_2 and COOH termini of the mature DTx-A (12), pTH1 (21), and pGA953 are shown in Figure 1. As compared to DTx-A the nucleotide sequence in pGA953 differs for two of the amino acid residues and there are also five additional codons on the NH_2 terminus while there are four additional codons on the COOH terminus.

Stable Transformation of Tobacco Cells with the DTx-A Gene

The binary vector pGA953 was transferred into *A. tumefaciens* containing a helper Ti plasmid (LBA4404) and tobacco leaf segments were cocultivated with the bacteria. Transformants were selected on shoot induction medium containing kanamycin sulfate (Fig. 2). Many shoots and calli appeared on each leaf segment cocultivated with *Agrobacterium* harboring pGA642, while none emerged on most of those treated with the strain carrying pGA953 (Fig. 2A).

Similar and more quantitative results were obtained from cultured tobacco cells (Fig. 2B, Table I). Coinfection of suspension cells with a 1:1 mixture of *Agrobacterium* carrying pGA953 and pGA642 also resulted in a reduced transformation efficiency (Fig. 2B; Table I).

Transient Coexpression of DTx-A and Chloramphenicol Acetyltransferase in Tobacco Protoplasts

To obtain further evidence that expression of DTx-A gene is toxic to plant cells, we have studied the effects of transient expression of the toxin gene in protoplasts. Tobacco NT1 suspension cells were protoplasted and the pGA953 DNA was coelectroporated with pGA865 DNA carrying the chloramphenicol acetyltransferase (*cat*) reporter gene. The plasmid pGA865 is a high copy number derivative of pGA658 (3) harboring the *cat* gene which is under the control of the nopaline synthase promoter. Results shown in Figure 3 demonstrated that the level of *cat* expression from the pGA865 diminished with increasing amount of pGA953 DNA. For example, coelectroporation of 4 μg of pGA865 and 10 μg of pGA953 (one DTx-A molecule per one *cat* molecule) reduced the CAT activity by more than twofold. Such effects were not observed when the *cat* reporter molecule was introduced with the promoter-less DTx-A molecule (pGA968) or with pTH1.

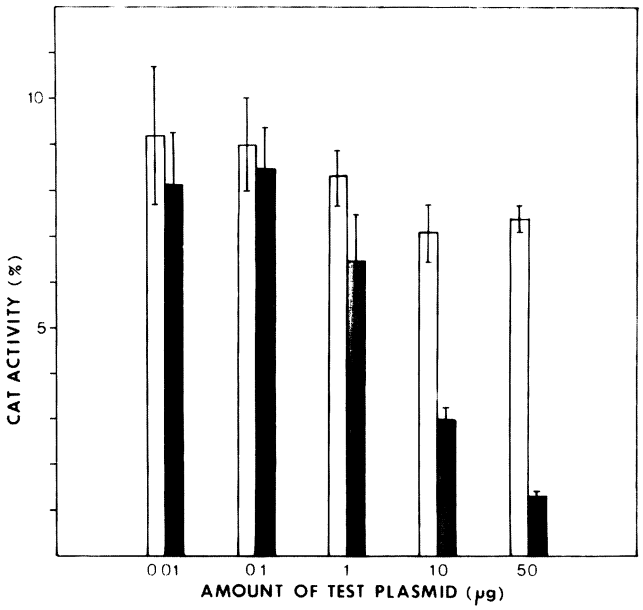

Figure 3. Transient assay of the DTx-A gene expression. Inhibition of transient CAT expression (expressed as percentage of conversion of labeled chloramphenicol) from pGA865 (carrying the *nos-cat* fusion) in NT1 protoplast-derived cells by coelectroporation with plasmids containing the DTx-A construct. Four micrograms of pGA865 was coelectrophorated with the indicated amount of DTx-A plasmids; pGA968 promoterless construct (white bar) or CaMV 35S-DTx-A construct pGA953 (shaded bar). Standard deviation is shown as a solid line on the top of each bar.

Table I. *Coinfection of Tobacco NT1 Cells with Agrobacterium Strains Carrying Binary Vector with or without the DTx-A Gene*

The values shown are the mean values obtained from duplicate plates in three separate experiments.

Binary Vector	No. of Kanamycin Resistant Colonies per Plate ± SD	Frequency[a]
		$\times 10^5$
pGA642	118.0 ± 55.1	1690.5
pGA953	0.3[b]	1.1
pGA642 + pGA953	18.7 ± 11.6	267.9

[a] The frequency was determined as the fraction of the number of kanamycin resistant colonies per plate and the number of plated suspension cell clusters that underwent the cocultivation treatment. [b] No SD was determined for the following figures: 1; 1; 0; 0; 0; 0.

Construction of Promoter-Probe Vector

To facilitate the use of the DTx-A as a suicide marker in plants, we have developed a promoter-probing molecule pGA987 from pGA953 by removing the CaMV 35S promoter (Fig. 1). This binary Ti plasmid vector contains the promoterless DTx-A coding region followed by the terminator of the T-DNA gene 7. There are three unique restriction endonuclease sites (*Hin*dIII, *Xba*I, and *Hpa*I) in front of the DTx-A gene where a variety of promoter fragments can be inserted.

DISCUSSION

We have demonstrated the toxicity of the diphtheria toxin chain A gene in plant cells by both stable and transient expression assays. The presence of the functional DTx-A gene in a binary plant expression vector significantly reduced the stable transformation frequency in both leaf segments and cultured tobacco cells, indicating that its expression during the process of transformation is detrimental to the establishment of stable transformants (18). Cocultivation of suspension cultured cells with an equal number of cells from two

different *Agrobacterium* strains carrying either a binary vector conferring kanamycin resistance (pGA642) or the binary vector with the DTx-A gene in addition the selectable marker (pGA953) greatly diminished the recovery of kanamycin resistant clones. The reduced transformation was probably due to the cotransfer of the two different T-DNAs to the same plant cell as observed previously (22).

Intracellular production of DTx-A should result in DNA degradation and inhibition of protein synthesis and therefore of gene expression. Indeed, the electroporation of the chimeric DTx-A gene into tobacco cells lowered the level of transient expression of the coelectroporated *cat* gene in a concentration dependent fashion. Such effects were not observed with the DTx-A genes which lack plant promoter, supporting the conclusion that the inhibition observed with pGA953 was due to active DTx-A production in the plant cells.

The chimeric DTx-A construct is a negative selection gene and is more potent than the amido hydrolase gene *2* of *Agrobacterium* transfer(T)-DNA which has been proposed for the elimination of a specific group of cells or tissues and for fundamental studies on gene inactivation (9). Eukaryotic cells can be extremely sensitive to DTx; it was reported that a single molecule of the polypeptide was lethal to a mammalian cell (30). We also have evidence that plant cells are very sensitive to the action of DTx-A since an attenuated mutant DTx-A gene under the control of the CaMV 35S promoter was still very active (data not shown). DTx-A requires no externally added substrates which themselves may be toxic to plant cells. We have observed that the substrates (indole-3-acetamide and 1-naphthalenacetamide) of the amido hydrolase are very toxic to *Arabidopsis thaliana*. Furthermore, the products of amido hydrolase diffuse to surrounding cells. The action of DTx-A is cell autonomous, that is the toxicity is localized to specific cells because the gene product is an intracellular toxin which is not active once outside the cell. A chimeric *RAS2* gene from yeast also had a "killer" effect in *Nicotiana plumbaginifolia*; however, the manifestation of toxicity was species-specific and depended on other conditions (16).

The cell autonomous action, potent toxicity, and broad spectrum including monocots (5) and dicots (tobacco) make the chimeric DTx-A gene potentially useful for studying cell-lineage relationships and for analyzing cellular interactions during development (for mammalian example, see ref. 26) by obtaining transgenic plants carrying a fusion of a cell-, tissue-, or developmental-specific promoter and the DTx-A coding region. The promoter probe binary plant vector pGA987 provides convenient restriction sites for insertion of promoter fragments in front of the DTx-A gene.

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