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Arrest of embryo development in *Brassica napus* mediated by modified *Pseudomonas aeruginosa* exotoxin A

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Abstract

Intracellularly expressed cytotoxins are useful tools both to study the action of plant regulatory sequences in transgenic plants and to modify plant phenotype. We have engineered a low mammalian toxicity derivative of *Pseudomonas aeruginosa* exotoxin A for intracellular expression in plant cells by fusing the ADP ribosylating domain of the exotoxin gene to plant regulatory sequences. The efficacy of exotoxin A on plant cells was demonstrated by transient expression of the modified exotoxin gene in tobacco protoplasts: the exotoxin gene inhibited the expression of a co-electroporated β-glucuronidase gene. An exotoxin with an introduced frameshift mutation was also effective at inhibiting β-glucuronidase expression in the transient assay; the activity of the frameshifted gene was presumably a result of frameshifting during translation or initiation of translation at a codon other than AUG. When fused to napin regulatory sequences, the exotoxin gene specifically arrested embryo development in the seeds of transgenic *Brassica napus* plants concomitant with the onset of napin expression. The napin/exotoxin chimeric gene did not have the same pattern of expression in tobacco as in *B. napus*; in addition to exhibiting an inhibition of seed development, the transgenic tobacco plants were male-sterile.

Introduction

The fusion of cytotoxin genes to tissue-specific regulatory sequences can result in the programmed destruction of specific cell types during development. Cytotoxins used in this manner are powerful tools to study the tissue specificity and timing of regulatory sequences because tissue ablation can be directly observed. The arrest of tissue development can also be used to study cell lineages and cell to cell interactions.

In transgenic mice, expression of the diphtheria toxin A chain (DT-A) gene in pancreatic acinar cells resulted in mice lacking normal pancreatic development [27]. Targeting DT-A to growth hormone expressing cells of the pituitary resulted in dwarf transgenic mice [3]. Recombinant DT-A and ricin genes, fused to murine crystallin reg-

ulatory elements, were used to study the optic tissue development of transgenic mice [4, 20]. RNases and DT-A have been fused to tapetum-specific regulatory elements from tobacco to study anther development and to engineer male sterility in plants [19, 23].

Pseudomonas aeruginosa exotoxin A is a powerful toxin which inhibits protein synthesis by ADP ribosylating mammalian protein elongation factor EF-2 [16]. Three functional domains have been identified: (1) a cell recognition domain; (2) a domain mainly involved in translocation of the toxin into mammalian cells; and (3) a domain catalyzing the ribosylation of EF-2 [5, 15]. Originally, domains Ib and III were thought to be necessary for catalytic activity [15]; however, subsequent work showed only domain III to be necessary [31]. Toxin molecules which have domains I and II deleted retain full ADP ribosylation activity while exhibiting little or no cytotoxicity to mammalian cells [15]; such molecules are good candidates for tissue ablation experiments.

Translation of an exotoxin encoding mRNA by a eukaryotic cell is expected to be lethal. One or few exotoxin molecules are sufficient to inactivate translation in a single cell and therefore to kill the expressing cell. In addition to causing lethality, this property has important implications for the heterologous expression work presented below. First, it is impossible for this product to accumulate in a eukaryotic cell since the first exotoxin molecule(s) to be synthesized will stop subsequent translation of more exotoxin mRNAs. Secondly, the amount of exotoxin present in an expressing tissue would be extremely low, probably below the limits of immunological detection. Thirdly, the addition of mRNA encoding exotoxin A to an *in vitro* translation system will inhibit subsequent *in vitro* translation, and therefore, only a few molecules of exotoxin A protein would be produced.

Many plant seed storage proteins are expressed in a tissue-specific fashion. Several researchers have introduced legume seed storage genes (both the coding sequences and flanking 5′ and 3′ regions) into transgenic tobacco or petunia, and demonstrated that they carry the regulatory signals necessary to confer tissue-specific expression in the heterologous system [1, 26, 32, 33]. These characteristics of seed storage protein genes make their regulatory regions suitable for fusion to cytotoxin genes to study gene regulation and the effect of cell ablation on seed development.

Napin is a storage protein synthesized in developing embryos of *Brassica* species; it comprises 20–30% of the total protein of mature seeds [9]. Northern analysis of developing embryos detected no napin mRNA at 15 days after anthesis (dpa); low levels of napin mRNA were first detected at 18 dpa, and the mRNA level peaked at 27 dpa [10]. DNA sequences flanking napin have been cloned, fused to β-glucuronidase (GUS), and been shown to direct GUS expression in developing embryos, but not in leaves of transgenic *B. napus* (J. Kridl *et al.*, in press).

In this manuscript, we describe the engineering of *Pseudomonas* exotoxin A for intracellular expression in plants by cloning the domain which catalyzes ADP ribosylation of EF-2 into plant expression cassettes. Using an electroporation assay with a constitutively expressed exotoxin, we demonstrate the efficacy of the modified exotoxin at inhibiting gene expression in tobacco cells. An exotoxin gene which contains a frameshift mutation is nearly as toxic as the in-frame exotoxin gene in the electroporation assay, presumably due to aberrant translation during synthesis of the exotoxin protein. By expressing the exotoxin gene under the control of napin regulatory elements, we are able to selectively abort embryo development in transgenic *B. napus*. In transgenic tobacco, the napin/exotoxin construct confers male sterility in addition to embryo abortion.

Materials and methods

Constructs

A *Bgl* II to *Eco* RI fragment (nucleotides 1488–2760 [12]) of *Pseudomonas aeruginosa* PAK exotoxin A (*toxA*) from pMS150A [21] was cloned into the *Bam* HI and *Eco* RI sites of pCGN566 (pUC19 polylinker in a plasmid conferring chloramphenicol resistance [7]) to give pCGN1590.

Linkers were synthesized to approximate the functional deletion of *toxA*, JH17 [15]; the exotoxin we used contained 25 amino acids of domain Ib and all of domain III. The synthetic linkers also contained a plant consensus translational start codon [22] as well as convenient restriction sites for cloning. In addition, another linker was synthesized which was identical except that it contained an additional base pair designed to cause a frameshift mutation in the protein synthesized (Fig. 1). These linkers were ligated to an *Apa* I digest of pCGN1590.

pCGN7394 contains a hybrid CaMV 35S and mannopine synthase promoter (Mac promoter [8]) driving the *Escherichia coli* GUS gene with a mannopine synthase (mas) 3′ region to direct termination and polyadenylation. The products of ligations of pCGN1590 to the linkers were cut with *Eco* RV and *Eco* RI and the exotoxin genes were cloned into the *Sma* I and *Eco* RI sites of pCGN7394 to replace the GUS gene with exotoxin genes (Fig. 1). After sequencing across the synthesized linkers, these plasmids were designated pCGN7150 (frameshifted exotoxin) and pCGN7151 (in-frame exotoxin).

Plasmid pCGN1808 contains a napin expression cassette derived from *Brassica campestris*. The cassette is composed of 1751 nucleotides of the region 5′ to the napin transcription initiation site and 1255 nucleotides from the 3′ region; the 5′ and 3′ regions are separated by a linker containing *Sal* I, *Bgl* II, *Pst* I, and *Xho* I cloning sites (J. Kridl *et al.*, in press). In order to clone the exotoxin genes into the napin cassette, they were first shuttled into Bluescript II (Stratagene) as *Hin*d III to *Eco* RI fragments from pCGN7150 and pCGN7151. The *Hin*d III sites were opened, filled in with Klenow, and religated to eliminate the sites for ease of subsequent cloning steps. The frameshifted exotoxin and the in-frame exotoxin genes were then cloned out of Bluescript as *Sal* I to *Pst* I fragments, and ligated to the napin cas-

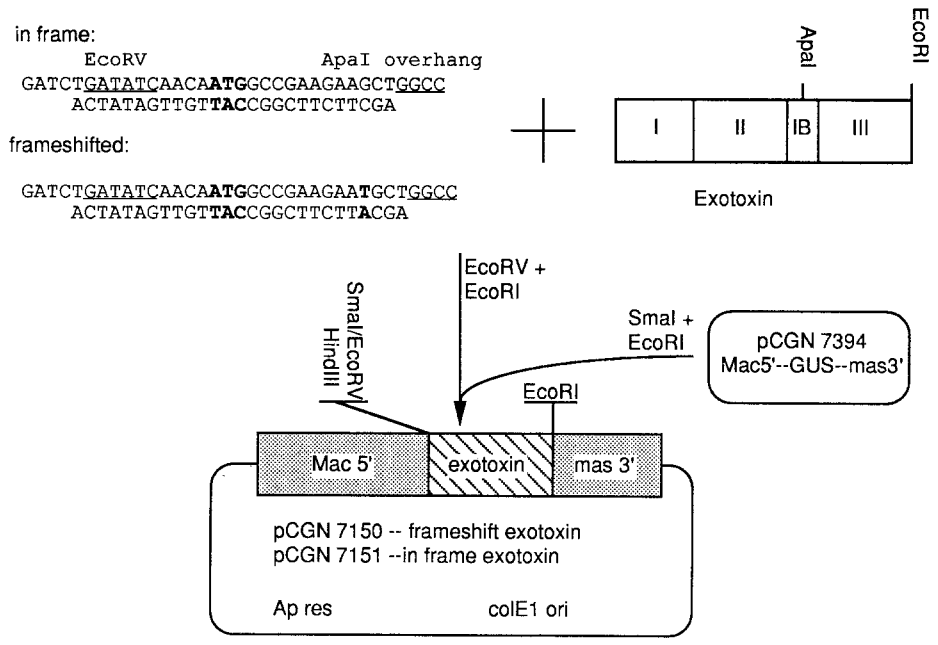

Fig. 1. Construction of Mac/exotoxin genes. In the upper left, the synthetic linkers used to construct the exotoxin genes are shown. The underlined DNA sequences are restriction sites or overhangs described in Materials and methods. The bold-face sequences are the methionine codons used to initiate translation and the nucleotides inserted to generate the frameshifted exotoxin gene. In the upper right the exotoxin gene is diagrammed. The roman numerals refer to structural domains of the exotoxin [15].

sette plasmid digested with the same enzymes. An anti-sense exotoxin was created by cloning the *Sal* I to *Pst* I fragment of the in-frame exotoxin into *Xho* I to *Pst* I sites of pCGN1808. These napin exotoxin constructs were cut with *Hin*d III and ligated to *Hin*d III-cut binary plasmid pCGN1578 [24] to yield pCGN7170 (frame-shifted exotoxin), pCGN7171 (in-frame exotoxin) and pCGN7172 (anti-sense exotoxin).

Transformation of tobacco and Brassica

Transformation of *B. napus* cv. Westar was performed as described by Radke *et al.* [30]. Transformation of tobacco was described by Horsch *et al.* [14].

Electroporation

Young leaves were selected from 3–4-week old *Nicotiana tabacum* (cv. Xanthi) plants grown axenically in MS medium (Gibco) supplemented with 30 g/l sucrose, 1.0 mg/l indoleacetic (IAA) and 0.15 mg/l kinetin, pH 5.55. Leaf halves without midribs were vacuum-infiltrated to 40 Pa with a 6% sorbitol solution containing 0.04% pectinase (Pectolyase Y-23, Seishin Pharmaceutical Co., Japan), 0.45% cellulase (Onozuka RS, Yakult Pharmaceutical Industry Co., Japan), and 0.5% potassium dextran sulfate, pH 5.55. The plant material digested for 3–4 h while gently shaking at 50 rpm. Protoplasts were separated from debris by passing the macerate through a 52 μm mesh nylon screen. Protoplasts were pelleted by centrifugation at 150 × *g* for 5 min and washed 3 times using a 7% sorbitol solution containing 1 mM CaCl and 10 mM HEPES. Protoplasts were suspended at a density of 2–3 $\times 10^6$ in an electroporation buffer containing 6% sorbitol, 10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$ pH 7.1 and a carrier DNA (pUC 19) concentration of 175 μg/ml.

Electroporation was carried out in electroporation cuvettes as described by Potter *et al.* [29]. 50 μg of each plasmid DNA was used in each electroporation reaction. The appropriate plasmid DNAs were added to 1 ml aliquots of the protoplasts in electroporation buffer and a single pulse of 300 V (600 V/cm field strength) was discharged from a 1250 μF capacitor. Following electroporation, protoplasts were transferred to 9 ml of MS medium supplemented with 30 g/l sucrose, 0.6 mg/l naphthalene acetic acid, 0.2 mg/l 2,4-dichlorophenoxyacetic acid, 0.8 mg/l kinetin and 5.5% sorbitol pH to 5.55, and allowed to incubate at 25 °C in the dark. Samples were taken at different time points and harvested by centrifugation at 50 × *g* for 8 min; the supernatant was discarded and pellets were frozen in liquid nitrogen and stored at −70 °C. The electroporated protoplasts were analyzed using the fluorometric assay for GUS activity described by Jefferson [17].

NPTII activity assay

Embryos were dissected from their seed coats and placed separately in microtiter dish wells containing water. These were kept on ice until assayed. Embryos were ground in Eppendorf tubes containing 20 to 40 μl of extraction buffer [29]. The pestle was then rinsed with 20 μl of fresh extraction buffer. The rinse was pooled with the original extract and kept on ice until all samples were processed. Between samples, the pestle was cleaned with water, then 95% EtOH, and then rinsed with water again. The extracts were centrifuged for 15 s at 4 °C to remove cellular debris. 10 μl of extract was assayed with and without the substrate, neomycin sulfate. NPTII assays were performed as described by Radke *et al.* [29].

Pollen viability staining

Pollen from tobacco plants was suspended in 50 μl of 0.5 M sucrose. 5 μl of 0.4 mg/ml fluorescein diacetate was added to the pollen suspension and the pollen grains were observed under a fluorescence microscope.

Kanamycin seed germination assay

Tobacco seeds were germinated on 0.8% agar plates containing MS salts (Gibco) supplemented with kanamycin at 400 μg/ml. After 2 weeks the seedlings were scored for resistance to kanamycin; resistant seedlings were green, while susceptible seedlings were bleached white.

Results

Cloning the exotoxin gene

Deletion analysis of exotoxin A by Hwang *et al.* [15] revealed that a deleted exotoxin protein, containing about 20 animo acids of structural domain Ib in addition to domain III, retained full catalytic activity at ribosylating mouse EF-2. This deletion mutant, JH17, contains no cell recognition or membrane translocation domains, making it a good candidate to express intracellularly. We designed a linker to duplicate the animo acid sequence of JH17. The linker, shown in Fig. 1, contains a plant consensus translational start site [22] about its initiator AUG codon. An *Eco* RV site, a *Bam* HI overhanging end, and an *Apa* I overhanging end were included to facilitate cloning. A similar linker containing an extra nucleotide in the coding region which shifts the protein sequence out of frame was used to construct a frameshifted exotoxin gene. The exotoxin genes were cloned into a Mac expression cassette [8], which confers a high level of constitutive expression in plant cells, in order to evaluate the efficacy of the exotoxin constructs by electroporation into tobacco protoplasts.

Both the in-frame and frameshifted exotoxin genes are toxic to tobacco protoplasts

We used an electroporation assay in tobacco protoplasts to determine whether the exotoxin constructs would produce protein in plant cells and whether such a protein would inhibit expression of a co-electroporated gene. We co-electroporated the Mac/exotoxin constructs, pCGN7150 and 7151, with a construct which directs expression of GUS, pCGN7394. Figure 2 shows that when pCGN7394 was electroporated by itself, the protoplasts actively expressed GUS. As a control, we co-electroporated a Mac/*aroA* [7] fusion with pCGN7394 (the *aroA* gene used is a mutated bacterial EPSP synthase gene which leads to tolerance of the herbicide glyphosate when expressed in transgenic plants). Since the Mac/*aroA* gene had little effect on GUS expression, we concluded that co-electroporating a second non-toxic gene under control of the Mac promoter does not markedly affect expression of GUS under the control of the same promoter. When the in-frame exotoxin gene (pCGN7151) was co-electroporated with pCGN7394, GUS activity was suppressed. This is presumably a result of the exotoxin inhibiting protein synthesis via ADP-ribosylation of EF-2. Surprisingly, when the frameshifted exotoxin gene on pCGN7150 was co-electroporated with pCGN7394, GUS activity

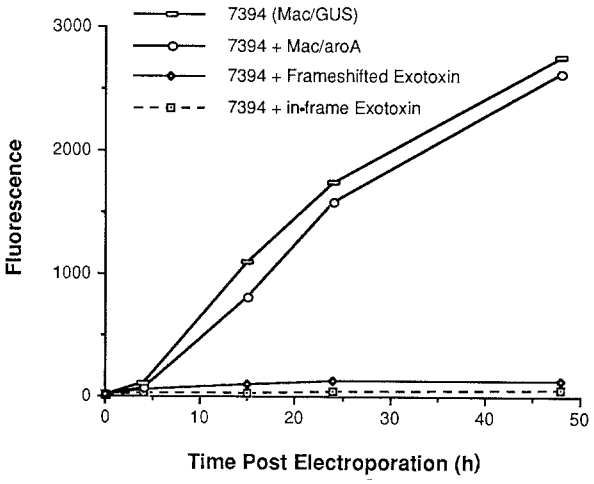

Fig. 2. GUS activity of electroporated protoplasts. GUS activity was measured in electroporated protoplasts at various time points up to 48 h after electroporation. Tissue extracts were incubated with the fluorometric substrate, MUG, for 24 h after which the fluorescence was determined. The line labelled 7394 represents data from protoplasts electroporated with pCGN7394, which contains the Mac/GUS gene fusion. The lines labelled 7394 + Mac/*aroA*, 7394 + Frameshifted Exotoxin, and 7394 + in-frame Exotoxin represent data from protoplasts co-electroporated with pCGN7394 and the respective *aroA* (control), frameshifted exotoxin, and in-frame exotoxin constructs.

was also suppressed. Presumably, an active toxin was produced despite the introduction of a frameshift mutation in the exotoxin gene.

Napin/exotoxin expression in transgenic tobacco

The exotoxin genes from plasmids pCGN7150 and pCGN7151 were cloned into the napin expression cassette of plasmid pCGN1808 (J. Kridl *et al.*, in press). The expression cassette of pCGN1808 contains about 1.75 kb of DNA sequence 5′ to the protein coding region of a *B. campestris* napin gene and approximately 1.25 kb of 3′ sequence. The in-frame exotoxin gene was also cloned in an antisense orientation in the napin cassette. The napin/exotoxin fusions were cloned into binary vector pCGN1578 [24]. Tobacco explants were co-cultivated with *A. tumefaciens* containing the binary vectors pCGN7170 (frameshifted exotoxin), pCGN7171 (in-frame exotoxin), and pCGN7172 (anti-sense exotoxin). Co-cultivation with pCGN7170 and pCGN7172 yielded normal tobacco shoots which subsequently rooted on medium containing kanamycin. Southern analysis showed most of these transgenic plants to contain the exotoxin gene (data not shown). Co-cultivation with pCGN7171 resulted in few shoots which were delayed in development. Most of the shoots did not root on kanamycin medium, and Southern analysis of plants derived from the shoots that did root showed that they did not contain the exotoxin gene (data not shown).

Transgenic tobacco plants transformed with pCGN7170 and 7172 appeared to be normal during vegetative growth. The plants transformed with the anti-sense exotoxin gene, pCGN7172, flowered and set seed normally. The transgenic plants transformed with the frameshifted exotoxin gene, pCGN7170, were male-sterile; only one plant of 13 independent primary transformants produced visible pollen. The transformant that produced pollen produced very little pollen when compared to untransformed tobacco plants, and only 9% of the pollen was judged viable by staining with fluorescein diacetate. In contrast, 82% of the pollen from untransformed *N. tabacum* cv. Xanthi stained with the fluorescent dye. None of the pCGN7170 transformants set self-pollinated seeds, not even the plant that produced some pollen.

Since the 7170 plants were male-sterile, we pollinated the plants with pollen from untransformed tobacco. The plants then set normal-looking seed pods. Seeds from these pods were germinated on medium containing kanamycin. Table 1 shows that a high proportion of the seeds failed to germinate, and all the seeds that germinated were kanamycin-sensitive, as indicated by bleaching of the cotyledons. Seeds that failed to germinate had a distinct collapsed appearance, and the intensity of this abnormality differed among transformants. Seeds from control plants, transformed with the anti-sense exotoxin gene (pCGN7172) germinated with high efficiency (>90%) and segregated for kanamycin resistance as normal trans-

Table 1. Segregation of tobacco seeds derived from crosses between untransformed tobacco and pCGN7170 transgenic plants.

Parent plant	Green seedlings[1]	Bleached seedlings[2]	Ungerminated seeds[3]
7170–4	0	ca. 300	339
–6	0	204	917
–8	0	336	650
–10	0	423	393
–11	0	260	444
–12	0	771	449
–13	0	466	378
–14	0	354	222
–15	0	637	261
–17	0	508	280
–18	0	688	344
–19	0	866	212
–24	0	456	520

[1] Tobacco seeds from transgenic plants containing the napin/exotoxin gene fusion were germinated on medium containing kanamycin. Green seedlings are kanamycin-resistant and indicate that the seedling inherited a transgene.

[2] Bleached seedlings are susceptible to kanamycin indicating that the transgene was not inherited.

[3] Seeds from a control plant which contained a single copy of the napin/anti-sense exotoxin gene had greater than 90% seed germination and segregated 1/1 for green and bleached seedlings.

genic plants (data not shown). This indicates that the T-DNA containing the frameshifted exotoxin prevented development of viable seeds, while gametes not inheriting the T-DNA could be fertilized and develop into viable seeds.

Napin-exotoxin expression in transgenic Brassica napus

B. napus explants were co-cultivated with *A. tumefaciens* containing the binary vectors pCGN7170 (frameshifted exotoxin under control of the napin regulatory elements), pCGN7171 (in-frame exotoxin), and pCGN7172 (anti-sense exotoxin). As observed in tobacco, co-cultivation with pCGN7170 and pCGN7172 yielded normal *Brassica* shoots which subsequently rooted, while co-cultivation with pCGN7171 resulted in few shoots which were delayed in development and did not root on kanamycin.

The transgenic plants transformed with pCGN7170 and 7172 appeared normal throughout vegetative growth and flowering. The seeds from pCGN7172 transformants were normal. Figure 3A shows the phenotype of the seeds and pods from a typical pCGN7170 transformant; the seed pods appeared normal, but a majority of the seeds were shrivelled, while the remainder of the seeds were plump and normal-looking.

Table 2 shows the segregation analysis of the shrivelled seed phenotype in 18 transgenic plants. Nine plants had segregation ratios consistent with the 3:1 ratio expected if the shrivelled seed phenotype was caused by a single dominant gene and the parents were heterozygous. Four plants had ratios consistent with the 15:1 ratio expected if two independent loci were segregating for the gene.

Since it is difficult to directly detect the protein product of the exotoxin gene when it is expressed in a eukaryotic cell (see Introduction and Discus-

Table 2. Segregation of shrivelled seed from self-pollinated transgenic *B. napus*.

Parent 7170–	Shrivelled seeds	Plump seeds	% Plump seeds	χ^2 3:1[2]	χ^2 15:1[2]
1	123	38	24	0.17	82.74
2	181	38	17	6.83	46.06
3	80	16	17	3.56	17.78
4	240	111	32	8.21	385.68
5	75	22	23	0.28	44.69
6	218	4	2	63.72	7.50
7	100	24	19	2.11	36.34
8	125	46	27	0.33	124.45
9	155	52	25	0.00	125.81
10	18	2	10	2.40	0.48
11	153	71	32	5.36	247.54
12	273	97	26	0.29	251.73
14	265	19	7	50.78	0.09
16	211	70	25	0.00	167.00
17	sterile				
18	163	7	4	39.54	1.32
19	224	11	5	51.75	0.99
20	2	58	97	164.36	837.14
21	161	18	10	21.32	4.42
Westar	4	144	97		

[1] Seeds harvested from these transgenic *B. napus* plants containing the napin/exotoxin gene fusion were scored for the shrivelled seed phenotype.

[2] χ^2 values are shown for the hypotheses that the segregation ratios are 3:1 and 15:1. A χ^2 value greater than 6.64 allows rejection of the segregation ratio with a 99% level of confidence, and a value greater than 3.84 allows rejection with a 95% level of confidence.

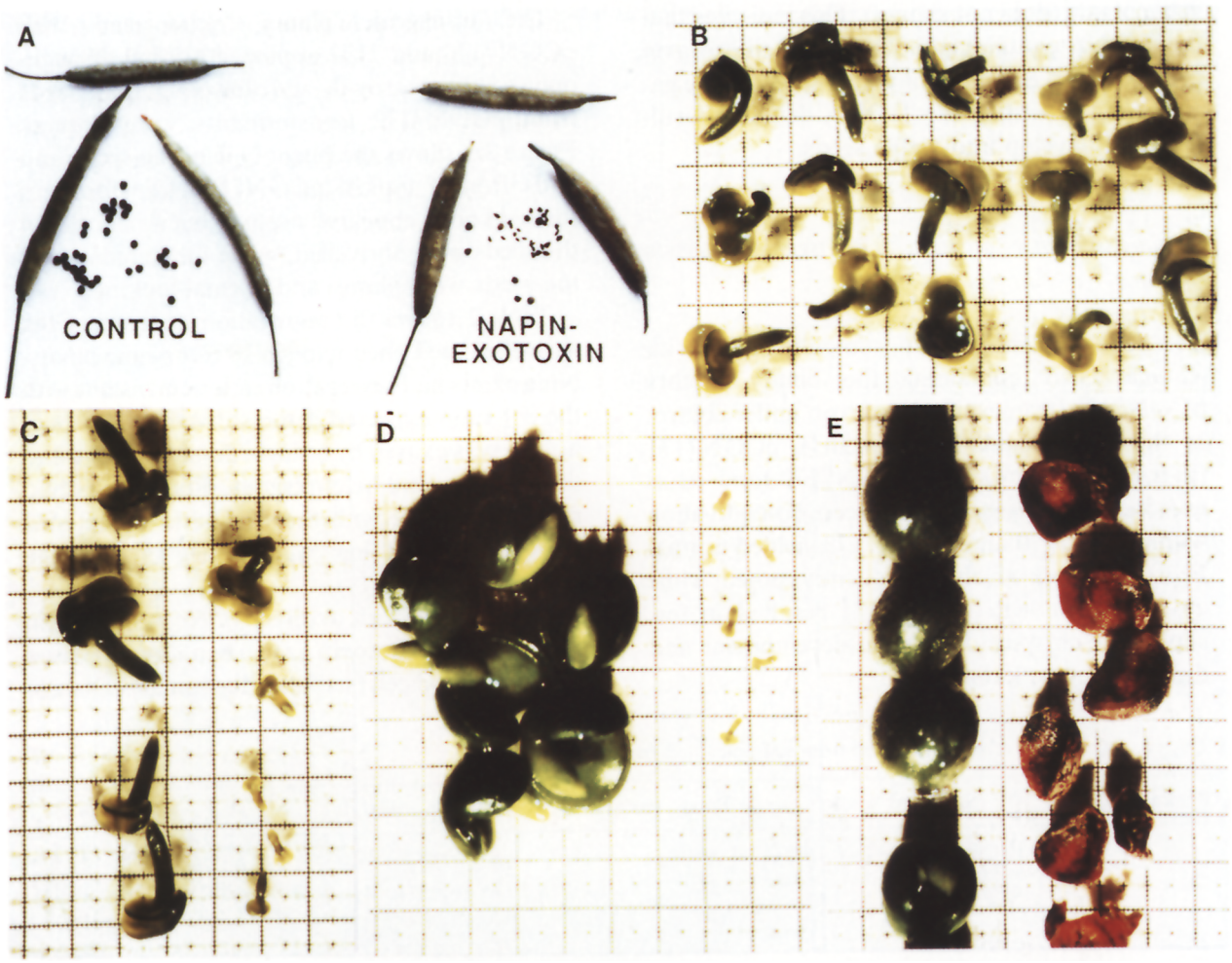

Fig. 3. Embryos and seeds from transgenic *Brassica* plants transformed with pCGN7170. A. Seed pods and seeds from untransformed Westar (control) and a transgenic 7170 plant (napin/exotoxin) are shown. A majority of the seeds from the napin exotoxin pod show the shrivelled phenotype. B. Embryos dissected from untransformed Westar at 17 dpa. The embryos were photographed on a 1 mm grid. C, D. Embryos dissected from a pCGN7170 transformant at 17 dpa (C) and 24 dpa (D). The embryos fall into two classes: those indistinguishable from the untransformed controls and those arrested in development. By 24 dpa the arrested embryos are shrinking and starting to discolor. E. Embryos dissected from the wild-type seeds of a pCGN7170 transformant at 28 dpa and whole collapsed seed of the shrivelled siblings. By 28 dpa the embryos were too small and desiccated to dissect, the seed coats had turned brown and the seed had collapsed into a concave lens shape.

sion for explanations), it was necessary to demonstrate the relationship between the shrivelled seed phenotype and the transgene by indirect experiments. We performed neomycin phosphotransferase (NPTII) assays on embryos dissected from immature seeds. If the shrivelled seed phenotype was caused by the exotoxin gene containing T-DNA, we expected to find that all the embryos from shrivelled seeds would inherit an exotoxin gene-containing T-DNA and express NPTII (the T-DNA contains an NPTII gene), while embryos dissected from normal plump seeds would not inherit a T-DNA and would not express NPTII. Figure 3C illustrates the size of the embryos that we assayed: embryos from the shrivelled seeds were about 1 mm in length with

0.2–0.4 mm wide cotyledons. Embryos from the normal seeds were 2–3 mm in length and the cotyledons were up to 1.5 mm in width. Figure 4 illustrates the results of the NPTII assays. Despite the small mass of the embryos dissected from the shrivelled seeds, we easily detected NPTII activity when individual embryos were assayed. We detected no NPTII activity in the embryos dissected from normal seeds. We assayed 22 embryos from normal seeds and 33 embryos from wrinkled seeds and found no breakage of linkage between the T-DNA and the shrivelled seed phenotype.

To further study the aborted seed phenotype, we tagged flowers of transgenic plants at pollination and dissected out the developing embryos at specific stages of embryo development. It was not possible to distinguish embryos of the seeds that would abort from those developing normally at 14 days after pollination. At 15 days after pollination some of the seeds appeared to have dimples. By 17 days after pollination, there were clearly two classes of embryos: those arrested and those continuing to develop (Fig. 3C). The arrested embryos never developed further; they eventually desiccated and turned brown (Fig. 3D). As seed development progressed the shrivelling of seed became apparent and some of the dimpled seeds dried prematurely. The seed coats of the shrivelled seeds appeared to develop normally; the formation of dimples and wrinkles were due to failure of the embryo to grow. By days 25 to 29 all of the shrivelled seeds were dried, while the plump, wild-type seeds were still green. Fig. 3E shows how the seed coats collapsed completely by the time the embryos were desiccated; we could no longer dissect the embryos from the shrivelled seeds at this stage of development. Within a seed pod we commonly found one or two seeds with embryos which were arrested at the heart stage of development. These seeds shrivelled earlier than the seeds which contained embryos aborting about 15–17 days after pollination.

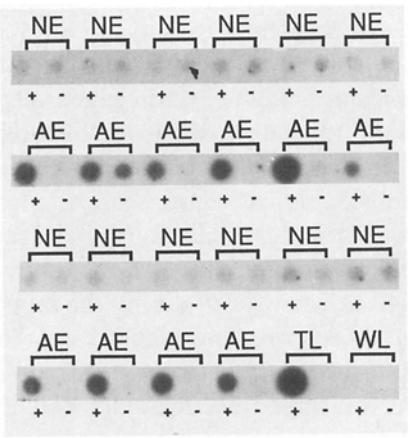

Fig. 4. NPTII assay of embryos from transgenic pCGN7170 *Brassica* plants. Embryos were dissected from seeds of a transgenic plant transformed with pCGN7170 and assayed for NPTII activity. + indicates wells to which the NPTII substrate neomycin was added, and − indicates wells which lacked the substrate. Embryos which have an increased signal in the + well compared to the − well were scored as exhibiting NPTII activity. NE refers to the normal embryos dissected from wild-type seeds, AE refers to the aborted embryos dissected from shrivelled seeds, TL refers to leaf tissue from the 7170 plant and WL refers to leaf tissue from an untransformed Westar plant.

Not all the plants exhibited embryo abortion at the same stage. Two of the plants had embryos that were arrested in development, but kept enlarging as the seeds developed. These plants had some seeds which were only partially shrivelled and contained embryos which were not fully developed. Two other plants had embryos arrested at the heart stage of embryo development. These plants had a reduced number of seeds in the seed pods, and the seed pods were shorter than wild-type pods. The pod phenotype and reduced seed set is characteristic of plants with reduced pollen fertility.

Discussion

We demonstrate that *Pseudomonas* exotoxin which inactivates the protein elongation factor EF-2 from mammalian cells also functions to inhibit development of plant cells. Presumably the mechanism of action is similar to that in mammals, where the exotoxin ribosylates plant EF-2. We observed that some of the plasmids that contained the in-frame exotoxin gene conferred al-

tered colony morphology on *E. coli*; the colonies grew very slowly and had a 'ghost white' color. Thus, the ADP ribosylating activity of the exotoxin may affect one or more *E. coli* proteins.

Expression of the frameshifted exotoxin gene

The frameshifted exotoxin was originally constructed as a negative control; we expected it to exhibit no cytotoxicity. This construct, instead, proved to be toxic both in the transient assay under the control of the CaMV 35S promoter and in transgenic plants under the control of the napin promoter. While there is no direct proof that the frameshifted exotoxin gene directs expression of the exotoxin protein, this is the simplest hypothesis to explain the cytotoxicity. The best evidence is provided by the electroporation experiments. The only available assay for exotoxin expression in a eukaryotic system is the arrest of translation as discussed in the Introduction. Figure 2 shows that a small but reproducible amount of GUS synthesis takes place for a short period after electroporation, but subsequent accumulation is blocked. This observation is consistent with the mode of action predicted for exotoxin A and does not support other generic toxicity models which would not be expected to abruptly terminate translation. Since exotoxin A would halt its own translation, the amounts of exotoxin present in expressing tissues are expected to be below the threshold of detection using an antibody against exotoxin A.

Examination of the exotoxin DNA sequence [12] shows that there are no methionine codons in frame with the exotoxin protein at which translation could initiate in the 'frameshifted' exotoxin. One possible explanation is that translation could initiate at a codon other than AUG. Yeast ribosomes can initiate translation at alternative codons with reduced efficiency [6, 35], and other initiation codons besides AUG codons can be used by certain mammalian viruses [2, 11, 13, 25]. Peabody [28] demonstrated that translation of dihydrofolate reductase could initiate at codons other than AUG both in *in vitro* translation reactions and *in vivo* in transformed monkey cells. A second hypothesis is that translational frameshifting occurs at low frequency in tobacco and *B. napus* cells to allow the frameshifted construct to produce active exotoxin. Frameshifting is known to occur during the expression of yeast Ty element genes and many retroviral gag and pol genes (for a review, see [18]). *Pseudomonas* exotoxin A and diphtheria toxin A both have similar mechanisms of action. It has been estimated that a single molecule of the DT-A fragment can kill a cell [34]. Therefore, a very low frequency of aberrant translation would be sufficient to explain the plant phenotypes we observed. Both napin and CaMV 35S are very strong promoters expected to produce hundreds to thousands of mRNA molecules per cell. If the efficiency of aberrant translation was one percent or less, it would be sufficient for the production of lethal amounts of exotoxin A. The most compelling argument for the production of exotoxin A is that any other model of toxicity (abnormal mRNA, fortuitous translation of another toxic protein) potentially applies to any gene. To date, hundreds of different genes have been expressed in plants without evidence of accidental toxicity.

The co-cultivation experiments demonstrated that the frameshifted exotoxin gene is less potent than the in-frame exotoxin gene. We were unable to recover transgenic plants with the in-frame exotoxin gene, however transgenetic plants with the less potent frameshifted exotoxin gene were obtained. We assume that the napin promoter is expressed at a low level during the regeneration process, thus preventing plants with the napin/in-frame exotoxin gene from developing. If the aberrant translation necessary for activity of the frameshifted exotoxin gene occurred at a very low frequency, viability of plants regenerated from pCGN7170 could be explained.

Tissue specificity of the napin/exotoxin gene fusion

In *B. napus* , the napin/exotoxin chimeric gene mimics the spatial and temporal expression pattern of the endogenous napin gene. Even the seed coats of aborted seeds developed normally indi-

cating that the tissue ablation caused by the exotoxin is cell autonomous: expression in one cell does not spill over into adjacent cells. The premature desiccation observed in the seed coats of the shrivelled seeds can be explained by the absence of a normal embryo. Northern analysis by Crouch *et al.* [10] showed that transcription of the napin gene in embryos begins between 15 and 18 dpa. When J. Kridl *et al.* (in press) fused napin regulatory regions to the GUS gene, GUS activity was detected by 17 dpa. Our results are in agreement with this timing of napin expression; in most transformants the inhibition of embryo development occurred between 15 and 17 dpa. A few of the transgenic plants containing the napin exotoxin fusion had different temporal expression patterns. It is unknown whether this is due to different levels of expression in different transformants. For example, transformants that abort earlier may have higher expression levels, while those with arrested embryos which continue to develop may have lower expression levels. It is also possible that these differences could be due to slight alterations of tissue specificity of the napin regulatory regions in different transformants.

In tobacco the napin/exotoxin gene results in both male sterility and seed abortion. Since pollen development is more sensitive to metabolic imbalances than most aspects of plant development, it is possible that there is also a low level of expression of the frameshifted exotoxin gene in other tissues besides the anther. After pollinating the transgenic plants with the frameshifted exotoxin with pollen from untransformed plants, a large number of seeds failed to germinate. The seeds that did germinate failed to inherit exotoxin-containing T-DNAs suggesting that the exotoxin gene is expressed in tobacco embryos. The contrast between the male fertility of *Brassica* plants transformed with the napin/frameshifted exotoxin and the male sterility of tobacco plants transformed with the same construct suggests that the napin regulatory sequences do not confer precisely the same tissue specificity in tobacco as they do in *Brassica*.

Understanding development in multicellular organisms will require the ability to dissect the processes of cell-to-cell communication. Physical removal, or ablation, of certain cell types would allow identification of their contribution to positional information of neighboring cells. Selective expression of a cell inhibitory gene is an effective alternative to physical removal and will prove a useful tool for plant molecular biology. Cellular ablation experiments depend on the availability of cell- and tissue specific-promoters. Even very low levels of background transcription can prevent the recovery of transformed plants by prematurely killing transformed cells. Our results describe an easy solution to this problem. The translational efficiency of the exotoxin A gene was reduced by a frameshift mutation resulting in the production of transgenic plants and specific developmental arrest of the target tissue.

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