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Inhibition of *Agrobacterium*-Induced Cell Death by Antiapoptotic Gene Expression Leads to Very High Transformation Efficiency of Banana

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The death of plant cells in culture following exposure to *Agrobacterium tumefaciens* remains a major obstacle in developing *Agrobacterium*-mediated transformation into a highly efficient genotype-independent technology. Here, we present evidence that *A. tumefaciens* exposure induces cell death in banana cell suspensions. More than 90% of embryogenic banana cells died after exposure to *A. tumefaciens* and cell death was accompanied by a subset of features associated with apoptosis in mammalian cells, including DNA laddering, fragmentation, and formation of apoptotic-like bodies. Importantly, these cellular responses were inhibited in cells expressing the animal antiapoptosis genes *Bcl-xL*, *Bcl-2* 3' untranslated region, and *CED-9*. Inhibition of cell death resulted in up to 90% of cell clumps transformed with *Bcl-xL*, a 100-fold enhancement over vector controls, approaching the transformation and regeneration of every “transformable” cell. Similar results using sugarcane, a crop plant known for recalcitrance to *Agrobacterium* transformation, suggest that antiapoptosis genes may inhibit these phenomena and increase the transformation frequency of many recalcitrant plant species, including the major monocot cereal crop plants. Evidence of inhibition of plant cell death by cross-kingdom antiapoptotic genes also contributes to the growing evidence that genes for control of programmed cell death are conserved across wide evolutionary distances, even though these mechanisms are not well understood in plants.

Additional keywords: necrosis.

Agrobacterium tumefaciens is a soilborne pathogenic bacterium that can be “disarmed” and modified to serve as a vehicle for gene transfer to plants. Genetic transformation is one of the most important crop improvement technologies and *Agrobacterium*-mediated transformation is one of the two primary methods. However, a large number of crop plant species are recalcitrant to *Agrobacterium*-mediated transformation. Even where this method is practical, it often is limited to certain cultivars. This has proven to be a major obstacle in developing an efficient transformation technology that can be adapted easily to a wide range of plant genera, species, and cultivars. Genetic improvement through gene transformation is very important for crops such as banana, where triploidy and sterility of most edible cultivars makes conventional methods extremely difficult. Sterility also precludes post-transformation transgene segregation or introgression through breeding, making it imperative to use transformation systems such as *Agrobacterium*-mediated transformation that result in low copy transgene integration.

The variation in response of plant tissues to *A. tumefaciens* has been attributed in part to differences in the ability of this bacterium to attach to plant cells or to differences in T-DNA transfer machinery (Lippincott et al. 1977; Nam et al. 1997; Yanofsky et al. 1985a and b). However, more significantly, cell death is observed in cultures of many plant cells following exposure to *A. tumefaciens*. *A. tumefaciens* triggers expression of many genes in the host cell, including components of plant defense machinery (Ditt et al. 2001; Veena et al. 2003). The modification of transformation parameters can increase the probability of stably transforming some recalcitrant cell types; however, cell death following *A. tumefaciens* infection still remains a significant limitation (Gelvin 2003). Tissue browning and necrosis following exposure to *A. tumefaciens* occurs in many monocot and dicot plants, including poplar (de Block 1990), grape (Deng et al. 1995; Perl et al. 1996; Pu and Goodman 1992), sorghum (Carvalho et al. 2004; Gao et al. 2005), wheat (Parrott et al. 2002), tomato, pepper, and lettuce (Van der Hoorn et al. 2000; Wroblewski et al. 2005). One report indicated that maize callus infected with *A. tumefaciens* undergoes a rapid, hypersensitive type of cell death and this response was suppressed by expression of two baculovirus genes, *p35* and *iap* (Hansen 2000). However, recovery of transformed plants was not significantly improved, reportedly because of the low efficiency of T-DNA transfer from *A. tumefaciens* to maize (Hansen 2000).

DNA laddering and formation of apoptotic-like bodies has been observed in plant tissues undergoing programmed cell death (PCD) following exposure to various biotic (Ryerson and Heath 1996; Wang et al. 1996) and abiotic (Katsuhara 1997; Ryerson and Heath 1996) stresses. Transgenic studies also have demonstrated that some of the features characteristic of mammalian apoptosis occur in susceptible tobacco plants infected with certain necrotrophic fungi and that constitutive expression of proteins from the *Bcl-2* gene family confers resistance to *Agrobacterium*-mediated transformation. Even where this method is practical, it often is limited to certain cultivars. This has proven to be a major obstacle in developing an efficient transformation technology that can be adapted easily to a wide range of plant genera, species, and cultivars. Genetic improvement through gene transformation is very important for crops such as banana, where triploidy and sterility of most edible cultivars makes conventional methods extremely difficult. Sterility also precludes post-transformation transgene segregation or introgression through breeding, making it imperative to use transformation systems such as *Agrobacterium*-mediated transformation that result in low copy transgene integration.

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H. K. Khanna and J.-Y. Paul carried out most of the experimental work under supervision from J. L. Dale, and H. K. Khanna wrote this article along with J. L. Dale, R. M. Harding, and M. B. Dickman.

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*The e-Xtra logo stands for “electronic extra” and indicates that supplemental material is available online. Details about the PCR analysis and five additional figures are published online.

tance to these fungi (Dickman et al. 2001). In tomato, expression of *Bcl-xL*, an antiapoptotic member of the *Bcl-2* family, and *CED-9*, a *Bcl-2* analogue from *Caenorhabditis elegans*, have been reported to improve plant survival under abiotic and biotic stresses (Xu et al. 2004). Animal apoptotic regulators have been shown to either induce or suppress cell death in transgenic plants exposed to stress (Katsuhara 1997; Ryerson and Heath 1996; Wang et al. 1996). Here, we report the use of negative regulators of mammalian apoptosis to suppress cell death in *A. tumefaciens*-transformed plants cells which resulted in remarkably high transformation frequency.

RESULTS

Agrobacterium induces cell death.

We previously have reported that banana embryogenic cell suspensions (ECSs) undergo browning and subsequently die following exposure to different strains of *A. tumefaciens* and, despite a very efficient T-DNA transfer protocol, the recovery of transgenic plants from these cells was comparatively low

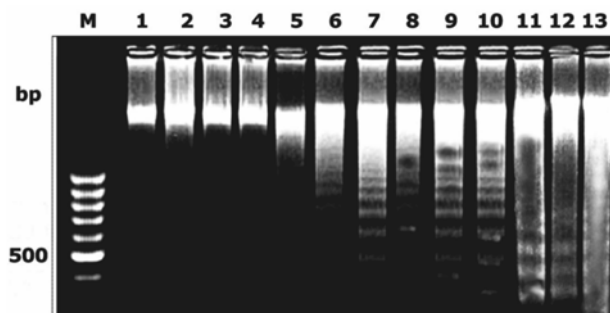


Fig. 1. *Agrobacterium*-induced nuclear DNA fragmentation in banana embryogenic cell suspensions. DNA isolated after 12 (lanes 2 through 4), 24 (lanes 5 through 7), 48 (lanes 8 through 10) and 72 (lanes 11 through 13) hours of *Agrobacterium* infection; lane 1: unexposed control. *Agrobacterium* inoculum densities used were optical density at 600 nm = 0.1: lanes 2, 5, 8, and 11; 0.5: lanes 3, 6, 9, and 12; and 1.0: lanes 4, 7, 10, and 13.

(Khanna et al. 2004). Heat-shock treatment of banana cells at 45°C for 5 min prior to transformation doubled the recovery of plants; however, considering that a large percentage of suspension cells transformed, the number of plants recovered was comparatively very low. Further studies then were undertaken to understand the cell death process and develop a strategy to enhance transformant recovery. ECSs from two banana cultivars (Grand Nain and Lady Finger) were exposed to *A. tumefaciens* AGL1 (a strain highly efficient for T-DNA transfer to banana cells) at a range of inoculum densities and for varying times to determine whether these factors affected the degree of cell death caused by this bacterium. Exposure of suspension cells to *Agrobacterium* spp. using a pre-optimized protocol (Khanna et al. 2004) at three different inoculum densities (optical density at 600 nm [OD_{600nm}] = 0.1, 0.5, and 1.0) indicated that *Agrobacterium* exposure reduced cell viability in a dose-dependent and time-dependent manner. Untransformed ECS clumps showed 98% viability but exposure to *Agrobacterium* spp. at an OD_{600nm} of 1.0 led to rapid cell death. Browning was visible on the fourth day and, by the seventh day, deep blue patches were visible in 90 to 95% of cell clumps, after trypan blue staining (Hou and Lin 1996), indicating the presence of dead cells. Control untransformed creamy white cell clumps maintained 92% viability under identical conditions. A lower inoculum (OD_{600nm} = 0.5) also induced a decline in cell viability but the degree of browning was reduced, as was the percentage of cell clumps (70 to 75%) that died by day seven. A further reduction in inoculum density (OD_{600nm} = 0.1) significantly reduced browning of cell clumps and only 35 to 40% of cell clumps showed cell death on day seven. There was no significant difference in the response of the two banana cultivars tested. It also was clear that, although low inoculum densities could be used to keep cell death relatively low, the corresponding reduction in T-DNA transfer resulted in low transformation efficiency (Khanna et al. 2004).

A. tumefaciens-induced cell death is apoptotic-like.

We then investigated whether cell death caused by *A. tumefaciens* was apoptotic-like or necrotic. A feature of mammalian apoptosis that differentiates it from necrotic cell death is the site-

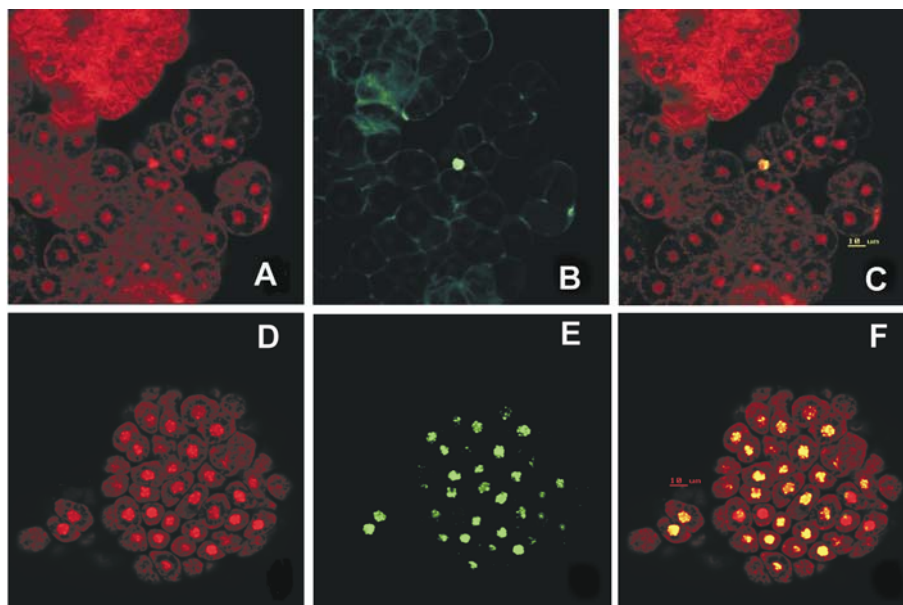


Fig. 2. dUTP nick-end labeling (TUNEL) assay shows that *Agrobacterium tumefaciens* induces DNA fragmentation in banana embryogenic cell suspensions. **A, B,** and **C,** Banana suspension cells not exposed to *Agrobacterium* spp. show only an occasional TUNEL-positive nuclei (**B**), but **D, E,** and **F,** cells exposed to *Agrobacterium* for 48 h at an optical density at 600 nm = 0.5 show a large number of TUNEL-positive nuclei (**E**). **A** and **D,** Propidium iodide-stained nuclei; **B** and **E,** the same nuclei with TUNEL labeling. **C** and **F,** Overlays of propidium iodide-stained and TUNEL-labeled nuclei ([**A** + **B**] and [**D** + **E**]) showing co-localization of staining.

specific cleavage of DNA by endonucleases (Eastman et al. 1994). Genomic DNA extracted at varying times from banana cell suspensions exposed to *A. tumefaciens* at a range of inoculum densities was analyzed by agarose gel electrophoresis. PCD-specific laddering of DNA was clearly evident in cells exposed to *A. tumefaciens*, whereas chromosomal DNA from control tissues remained intact (Fig. 1). A clear dose and time dependence was observed in cells exposed to *A. tumefaciens* inoculum at OD_{600nm} from 0.1 to 1.0. At the highest inoculum density (OD_{600nm} = 1.0), DNA laddering was observed within 24 h (Fig. 1, lane 7); however, at lower inoculum densities, laddering became prominent only after 48 h. DNA laddering was observed in all banana cells exposed to *A. tumefaciens* by 72 h (Fig. 1, lanes 11 to 13). These results indicate that the nuclear DNA is cleaved into nucleosomal fragments during the 2- to 3-day co-culture period. Importantly, the *A. tumefaciens* used in these experiments did not contain a binary vector; therefore, these results indicate that it was exposure to the bacterium rather than genetic transformation that caused cell death.

Another hallmark of apoptotic-like cell death is formation of apoptotic-like bodies containing fragmented DNA (Martin et al. 1994). The TdT-mediated dUTP nick-end labeling (TUNEL) method labels free 3'-OH groups of DNA and is used to detect specifically fragmented DNA and visualize apoptotic bodies in

situ (Gorczyca et al. 1993). We applied TUNEL staining and propidium iodide nuclear counterstaining to banana ECSs exposed to *A. tumefaciens*. Control cells incubated on co-culture media without exposure to *A. tumefaciens* contained intact nuclei (Fig. 2a) and TUNEL labeling of DNA was negligible (Fig. 2b and c). In contrast, cells treated with *A. tumefaciens* contained distorted nuclei (Fig. 2d) and more than 50% of these nuclei were TUNEL positive, indicating DNA fragmentation (Fig. 2e and f). TUNEL-positive nuclei were detected as early as 48 h after the start of co-culture with *A. tumefaciens*; however, cell death was not observed until 96 h past exposure. This indicated that nuclear DNA fragmentation preceded the loss of membrane integrity and confirmed that the observed DNA fragmentation was caused by apoptotic-like rather than necrotic cell death (Martin et al. 1994; O'Brien et al. 1997).

Inhibiting *Agrobacterium*-induced PCD.

Banana ECSs were transformed with antiapoptosis genes from diverse sources to determine whether expression from these genes could inhibit *Agrobacterium*-mediated cell death and provide a survival advantage to transformed cells. Binary vectors pPTN254, pPTN255, pPTN261, pPTN396, and pPTN290, each containing the maize polyubiquitin-1 (Ubi-1) promoter controlling the expression of *Bcl-xL* (chicken), *Bcl-xL* G138A (a point, loss-of-function mutant of chicken *Bcl-xL*) 21, *CED-9* (from *C. elegans*), *Bcl-2* 3' untranslated region (UTR) (human) (Awada et al. 2003), and the *UidA* reporter gene encoding β-glucuronidase (GUS), respectively, were used to transform banana ECS. *Agrobacterium* cells carrying the *UidA* reporter gene binary vectors did not show any blue coloration following GUS staining, indicating that there was no transcription of the GUS gene from any cryptic bacterial promoter sequences in the maize Ubiquitin promoter.

To prevent overgrowth of *Agrobacterium* in selection cultures, transformation experiments were carried out at OD_{600nm} = 0.5. The transformation protocol used (Khanna et al. 2004) was very efficient in T-DNA transfer to banana ECS, and banana suspension lines used were highly embryogenic. Approximately 90 to 95% of suspension cells showed *UidA* gene expression on day three of co-culture. However, *UidA* expression dropped to 30% by day seven, accompanied by browning of cell clumps. Similar observations were made with the binary vector carrying the *Bcl-xL* G138A gene; by day 10, cell viability had dropped to 10% in both treatments. In contrast, banana cells transformed with *Bcl-xL*, *CED-9*, and *Bcl-2* 3' UTR continued beyond seven days without displaying any significant browning. On day 10, cell viability in these cultures was assessed at 85, 82, and 78%, respectively, indicating that transient transgene expression early during the co-culture period had a cytoprotective effect sufficiently strong to protect most of the transformed cells.

To confirm this effect, transformed cells were subjected to TUNEL assay on day three of co-culture. Banana ECSs transformed with *Bcl-xL* G138A (Fig. 3d) or the *UidA* reporter gene (Fig. 3f) showed a comparable level of TUNEL-positive cells to cells exposed to AGL1 alone (Fig. 2e and f), confirming that the transformation process does not contribute significantly to cell death at this stage of co-culture. The number of cells displaying TUNEL-positive staining dropped significantly in cells transformed with *Bcl-xL* (Fig. 2f), *CED-9*, and *Bcl-2* 3' UTR (images not shown). The fragmentation of nuclei and the formation of micronuclei were observed at a high frequency in cells transformed with *Bcl-xL* G138A (Fig. 3c and d) or the *UidA* reporter gene (Fig. 3e and f) compared with the cells transformed with *Bcl-xL* (Fig. 3a and b), *CED-9*, or *Bcl-2* 3' UTR. Further, nuclear DNA isolated from untransformed and unexposed cells, and cells transformed with *Bcl-xL*, *CED-9*, or *Bcl-2* 3' UTR, was intact (Fig. 4, lanes 5-9), with no detectable

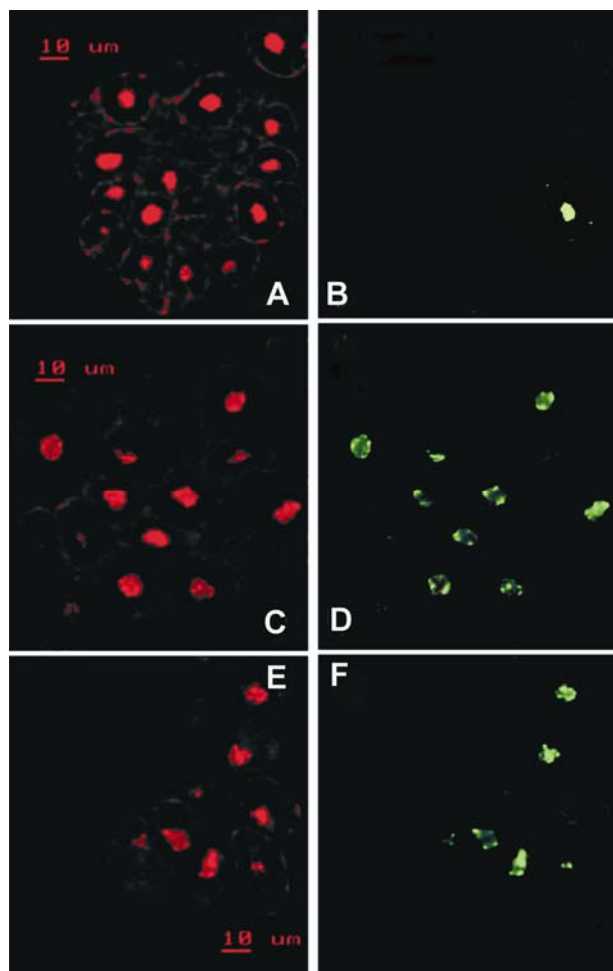


Fig. 3. dUTP nick-end labeling (TUNEL) assay shows that *Agrobacterium*-induced DNA fragmentation in banana embryogenic cell suspensions can be inhibited by antiapoptosis gene expression. Transformed after 48 h of exposure to *Agrobacterium* AGL1 at an optical density at 600 nm = 0.5; **A**, **C**, and **E**, propidium iodide-stained nuclei; and **B**, **D**, and **F**, the same nuclei with TUNEL labeling. Suspension cells harbor *Bcl-xL* (**A** and **B**), *Bcl-xL* (G138A) (**C** and **D**), and pPTN290 (β-glucuronidase) (**E** and **F**).

DNA laddering even at 72 h. In contrast, genomic DNA isolated from Grand Nain and Lady Finger cells exposed to AGL1, as well as cells transformed with the *Uida* reporter gene and *Bcl-xL* G138A, showed DNA laddering at 72 h (Fig. 4, lanes 1 to 4). The pattern of DNA laddering and TUNEL staining correlated with the pattern of visual browning and trypan blue staining in transformed cells, indicating that browning and loss of cell viability following *Agrobacterium* infection was a consequence of PCD that could be inhibited through expression of antiapoptosis genes.

Enhanced recovery of transformed embryos.

The most dramatic effect of antiapoptosis genes was seen on the recovery of transformed embryos from cells transformed with *Bcl-xL*, *CED-9*, or *Bcl-2* 3' UTR compared with ECSs transformed with *Bcl-xL* G138A (Fig. 5) or the *Uida* reporter gene. Untransformed control cells from Grand Nain and Lady Finger cell lines used for these experiments generated an average of 4,900 and 5,600 embryos per 50 mg of settled cell volume (SCV) of ECS, respectively, and these numbers were used as a baseline to assess the embryogenic potential of the cell lines (Table 1). Exposure of these cell lines to AGL1 without a vector reduced this number to 104 and 110 for Grand Nain and Lady Finger, respectively, in the absence of heat shock pretreatment. Heat shock pretreatment increased the recovery of embryos by almost twofold (Table 1). Similar results were obtained with ECSs transformed with the *Uida* reporter gene. These results indicated that exposure to *Agrobacterium* spp. was the major cause of poor embryo recovery from untransformed cells, and it is evident that lower transformant recovery from transformed cells was a direct consequence of this loss.

Grand Nain ECS transformed with *Bcl-xL* gave rise to an average of 4,630 embryos from four different experiments, and approximately 3,150 and 820 embryos were formed from ECS transformed with *CED-9* and *Bcl-2* 3' UTR, respectively, (Table 1). Comparable results were obtained with Lady Finger ECS (Table 1). These results suggest that expression from the antiapoptosis genes significantly inhibited cell death, leading to recovery of most of the transformed cells. Because the *Agrobacterium*-mediated transformation protocol used in this study was very T-DNA transfer efficient, coupled with an equally high recovery of transformed cells by abrogation of apoptotic-like cell death, recovery of transformed embryos was enhanced by up to a 50-fold using *Bcl-xL*. The other two antiapoptotic genes, *CED-9* and *Bcl-2* 3' UTR, also had a significant positive effect (Table 1).

Transgenic plants were resistant to *Agrobacterium*-induced cell death.

Four replicates of 100 embryos each from each vector transformation were transferred to regeneration media and trans-

formed embryos were regenerated into plantlets. *Bcl-2* 3' UTR transgenics began regenerating profusely after 4 weeks (at least 3 weeks before all other transformants) (Fig. 5e), including the untransformed controls. The conversion of embryos to plantlets was comparable for unexposed controls and embryos transformed with *Bcl-xL*, *CED-9*, and *Bcl-2* 3' UTR (Table 1). The plantlet conversion rate for *Agrobacterium*-treated but untransformed embryos or embryos transformed with a reporter gene and loss of function mutant was almost half that of embryos transformed with antiapoptosis genes, indicating that the antiapoptosis genes also had a positive effect on regeneration. The basis for this effect is not clear but may be related to the role of antiapoptosis genes in cell proliferation. Combined with a 50-fold increase in recovery of transformed embryos, the transformation efficiency could be improved by more than 100-fold by using the *Bcl-xL* gene compared with *Bcl-xL* G138A or the *Uida* reporter gene. Because the only difference between *Bcl-xL* and *Bcl-xL* G138A is the loss of function from a single nonsynonymous mutation, it can be concluded that this enhancement in transformation efficiency was due to the effect of *Bcl-xL* gene expression.

More than 50 transformants for each transgene were transferred to rooting media and characterized to i) identify independent lines harboring single copies of the transgene, ii) confirm the presence of transgene transcript, and iii) identify the proportion of escapes and clones. Only 2% of the regenerants were escapes, indicating that 98% of the transgenics were transformants. One or two copy-number, kanamycin-resistant trans-

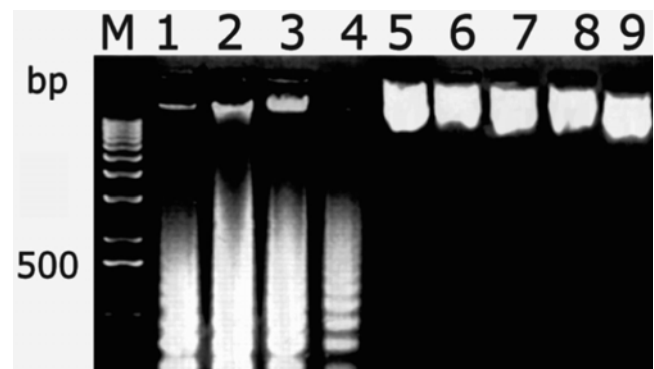


Fig. 4. Inhibition of *Agrobacterium*-induced nuclear DNA fragmentation in banana embryogenic cell suspensions. Genomic DNA isolated after 72 h of exposure to *Agrobacterium* (AGL1) at an optical density at 600nm of 0.5. Lanes 1, 2, 3, and 4: untransformed cells (cv. Grand Nain), untransformed cells (cv. Lady finger), pPTN290 (β -glucuronidase)-transformed cells, and *Bcl-xL* (G138A)-transformed cells, respectively; lanes 7, 8, and 9: *Bcl-xL*, *CED-9*, and *Bcl-2* 3' untranslated region transformed cells, respectively; lanes 5 and 6: untransformed Grand Nain and Lady finger cells, respectively, not exposed to *Agrobacterium* spp.

Table 1. Enhanced transformation in banana using antiapoptosis genes^a

Strain, vector ^b	Number of embryos		Plants per 100 embryos		Estimated plant number	
	Grand Nain	Lady finger	Grand Nain	Lady finger	Grand Nain	Lady finger
No agroinfection	4,900 ± 327	5,600 ± 373	56 ± 6	64 ± 7	2,744 ± 229	3,584 ± 299
AGL1/no vector*	162 ± 11	188 ± 13	22 ± 4	26 ± 3	36 ± 3	49 ± 4
AGL1/no vector	104 ± 7	110 ± 7	18 ± 2	19 ± 2	18 ± 2	21 ± 2
AGL1/pPTN290*	188 ± 13	224 ± 15	23 ± 5	28 ± 3	43 ± 4	63 ± 5
AGL1/pPTN290	102 ± 7	122 ± 9	21 ± 3	21 ± 2	21 ± 2	26 ± 2
AGL1/pPTN254	4,630 ± 242	5,249 ± 257	52 ± 6	62 ± 7	2,407 ± 157	3,227 ± 199
AGL1/pPTN255	96 ± 8	105 ± 8	19 ± 2	20 ± 2	18 ± 2	21 ± 2
AGL1/pPTN261	3,150 ± 183	2,730 ± 182	50 ± 5	59 ± 7	1575 ± 112	1,611 ± 134
AGL1/pPTN396	820 ± 140	1,060 ± 157	54 ± 7	63 ± 7	442 ± 65	667 ± 124

^a Data represents mean ± standard error from four replicates of 50 mg of settled cell volume of embryogenic suspension cells.

^b *Agrobacterium* strain and vector; * indicates embryogenic cell suspensions subjected to heat shock prior to transformation.

genic plants harboring the various antiapoptotic genes were selected for further analysis. Reverse-transcriptase polymerase chain reaction (RT-PCR) of selected plants confirmed transcription of transgenes in selected plantlets (Supplemental material). No developmental abnormalities were observed in the regenerated transgenic plantlets. Young, actively growing roots from plants transformed with antiapoptotic genes by *Agrobacterium* spp. were used to determine the cytoprotective effect of these transgenes on the stably transformed root cells.

Roots were tested for *Agrobacterium*-induced cell death by exposure to AGL1 using a high-density inoculum (OD_{600nm} of 1.0). The absence of TUNEL-positive nuclei in *Bcl-xL*-transformed root cells (Fig. 6f) compared with exposed but untransformed controls (Fig. 6d), and *Bcl-xL* G138A (Fig. 6h) and *UidA* reporter gene-transformed plants (Fig. 6j), demonstrated that the expression of antiapoptotic transgenes had a cytoprotective effect on differentiated banana cells and that root cells were protected from *Agrobacterium*-induced PCD. The absence of detectable DNA laddering in root cells from plants expressing *Bcl-xL*, *CED-9*, and *Bcl-2* 3' UTR after exposure to *A. tumefaciens* at a high inoculum density (OD_{600nm} of 1.0) (Fig. 7, lanes 2 to 4) compared with root cells from untransformed Grand Nain and Lady Finger plants (Fig. 7, lanes 6 to 7) and roots cells from plants transformed with *Bcl-xL* G138A and the *UidA* reporter gene (Fig. 7, lanes 8 to 9) further confirmed that these genes inhibited *A. tumefaciens*-induced cell death.

***Agrobacterium*-induced PCD is not banana specific.**

To examine whether *Agrobacterium*-induced PCD was specific to banana, we conducted similar experiments with sugarcane. Results indicated that *A. tumefaciens*-induced PCD also occurred in this transformation recalcitrant crop. Sugarcane transformation frequency was significantly enhanced (no transformants with *Bcl-xL* G138A to 35% with *Bcl-xL*), although some bleaching, possibly caused by high-level antiapoptosis gene expression from the maize polyubiquitin promoter, was evident in some plants.

DISCUSSION

Evidence presented here indicates that *A. tumefaciens* induced an apoptotic-like cell death in banana and sugarcane. There is

only one other published report that indicated that *A. tumefaciens* induces DNA laddering in maize (Hansen 2000), although "necrosis" following exposure to *A. tumefaciens* has been observed in many crops. Given that *A. tumefaciens* is a plant pathogen, such a response in a nonhost species is not entirely unexpected. Nonhost resistance is a poorly understood but common form of plant resistance, whereby all members of a plant species exhibit resistance to all members of a given pathogen species (Thordal-Christensen 2003). Nonhost resistance may or may not produce visible symptoms such as cell death (Mysore and Ryu 2004), and recent evidence has suggested that some genes and pathways are common to both nonhost and the more specific gene-for-gene incompatible resistance interactions. Thus, the line of demarcation between the two phenomena is less distinct. We suggest that *A. tumefaciens* induces PCD in nonhost plants, many of which previously have been reported to exhibit high levels of necrosis coupled with relatively low survival. Because many major crop species (such as wheat, maize, rice, sorghum, and sugarcane) are nonhost monocots and recalcitrant to *Agrobacterium*-mediated transformation, the demonstration that cell death inhibitors can have a dramatic impact on transformation efficiency in a nonhost plant species is highly significant. An efficient transformation system could play an important role in improvement of economically important crops such as banana, where genetic transformation is a very appropriate strategy for disease resistance and biofortification. Many of the major food crops continue to be difficult to transform using *A. tumefaciens* and, in many instances, the transformation protocols are very genotype dependent, requiring subsequent introgression of the transgenes into desirable genetic backgrounds. A high-efficiency, genotype-independent transformation protocol would be a major advance and would provide a significant boost toward both ongoing efforts to improve the agronomic properties of these crops and to the development of crops into "biofactories" for products of industrial importance. Although efficient T-DNA transfer and regenerable explants still remain prerequisites, inhibition of *A. tumefaciens*-induced cell death could greatly improve the frequency of transformation in recalcitrant crops where cell death has been the major limitation in recovery of transformants. Importantly, an efficiency of more than 90%, as demonstrated here with banana ECSs, suggests that selectable markers could be

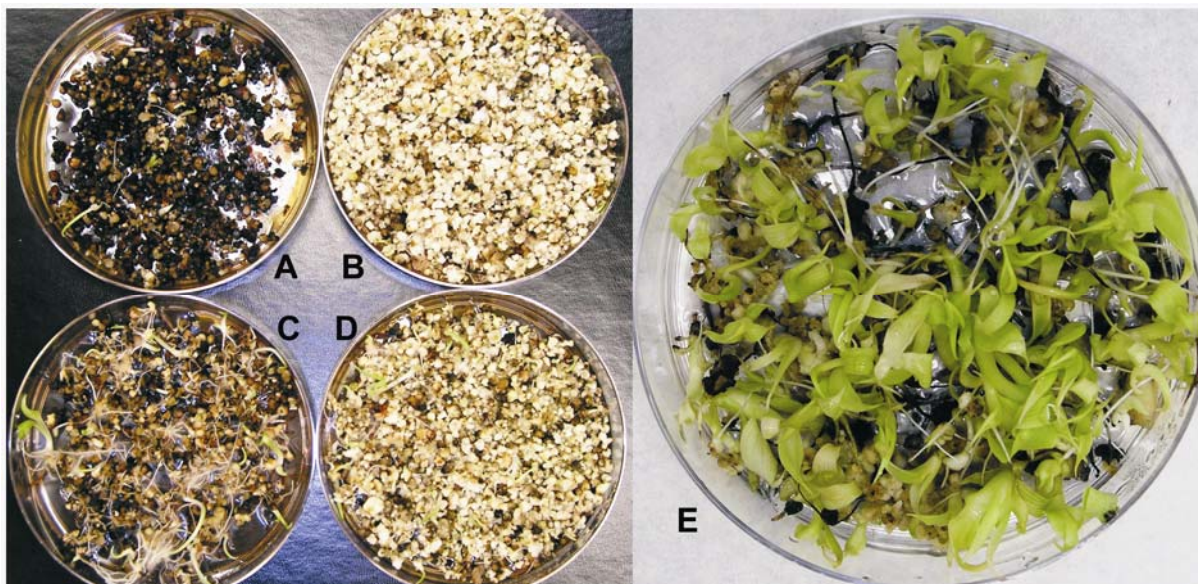


Fig. 5. Antiapoptosis genes enhance recovery of transformed embryos from banana cell suspensions. **A** through **D**, Embryogenesis and **E**, regeneration on selection media from banana cell suspension cultures transformed with **A**, *Bcl-xL* (G138A), **B**, *Bcl-xL*, **C** and **E**, *Bcl-2* 3' untranslated region, and **D**, *CED-9*.

unnecessary. This would be a very significant advance generally, but particularly for vegetatively propagated crops such as banana, where sexual crossing or subsequent segregation cannot be used for generating marker-free lines. Here, we have provided the proof of principle using constitutively expressing animal antiapoptosis genes; however, further studies are in progress to restrict the expression of these genes to an early culture phase, considering the potential toxicity of these genes in food. To address this issue, we also are isolating and assessing antiapoptosis genes from plants for their ability to mirror the impact of antiapoptotic genes of animal origin.

MATERIALS AND METHODS

Plasmid constructs.

Binary vectors pPTN254, pPTN255, pPTN261, pPTN396, and pPTN290 used for transformation had the maize polyubiq-

uitin-1 (Ubi-1) promoter controlling the constitutive expression of *Bcl-xL* (chicken), *Bcl-xL* G138A (one loss-of-function substitution at codon 138 of *Bcl-xL* protein), *CED-9* (from *C. elegans*), *Bcl-2* 3' UTR (human), and *Uida* reporter gene encoding GUS, respectively.

ECS and transformation.

ECSs of banana cvs. Grand Nain (*Musa* spp. AAA group) and Lady Finger (*Musa* spp. AAB group) were initiated, maintained, and transformed as described earlier (Khanna et al. 2004). Twenty-four-hour sucrose starvation was used for synchronization of suspension cells.

Cell viability test.

Loss of plasma membrane integrity was evaluated using trypan blue staining (Hou and Lin 1996). Control and *Agrobacterium*-infected samples (50 mg) were washed with suspension maintenance media and incubated for 1 h in incubation medium containing 0.4% trypan blue (in water, glycerol, and lactic acid; 1:1:1). After multiple changes of 50% glycerol-water to remove unbound dye, samples were observed under a light microscope. For visual assays, 200 cell aggregates were scored per treatment for positive staining, with three replicates per treatment. For quantitative assessment, bound trypan blue was extracted from 50 mg of SCV of stained ECS in 500 μ l of 50% methanol and 1% sodium dodecyl sulfate for 1 h at 50°C and quantified by measuring the absorbance at 595 nm.

DNA laddering.

DNA was isolated from cells infected with *Agrobacterium* spp. using a DNeasy Plant kit (Qiagen Inc., Valencia, CA, U.S.A.). Samples were thoroughly washed to remove *Agrobacterium* spp. prior to DNA isolation. For each sample, 5 μ g of DNA was loaded per lane, resolved on a 2% agarose gel at 60 V cm^{-1} for 4 h, and stained with ethidium bromide.

TUNEL assay.

Plant samples were fixed with 4% (vol/vol) formaldehyde in 0.1 M phosphate-buffered saline (pH 7.2) and permeabilized using 1% Triton X100 in 0.1% sodium citrate for 10 min. DNA was labeled by terminal deoxynucleotidyl transferase-mediated TUNEL using an in situ cell death detection kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. Samples were counterstained with propidium iodide at 0.5 $\mu\text{g ml}^{-1}$ and confocal images were obtained using a Leitz

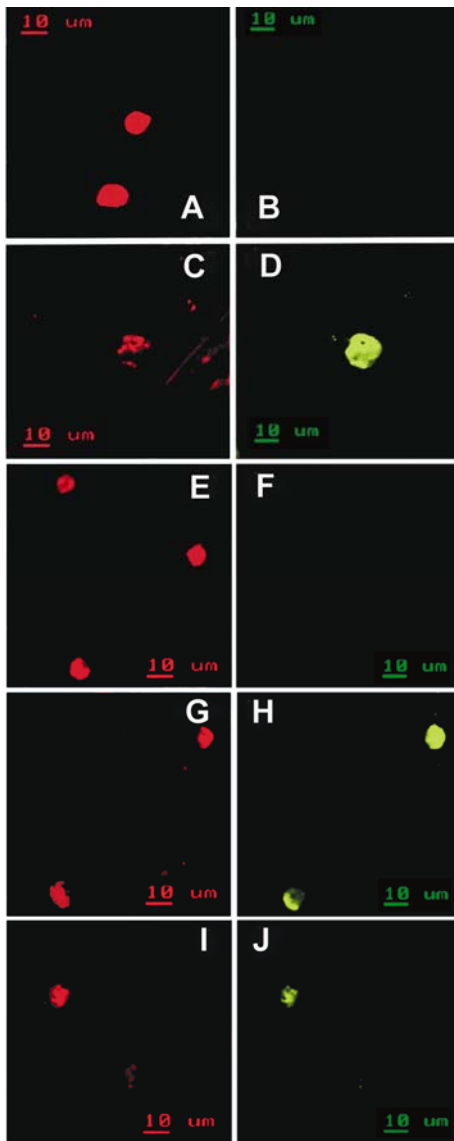


Fig. 6. dUTP nick-end labeling (TUNEL) assay indicates that *Agrobacterium*-induced fragmentation of nuclear DNA in banana root cells is inhibited in transgenics. A through D, Untransformed and E through J, transformed banana root cells before (A and B) and after (C through J) 48 h of exposure to *Agrobacterium* AGL1 at an optical density at 600 nm = 0.5. A, C, E, G, and I, Propidium iodide-stained nuclei; B, D, F, H, and J, the same nuclei after TUNEL labeling. Untransformed root cells prior to (B) and after (D) *Agrobacterium* infection. Transgenic root cells harboring *Bcl-xL* (E and F), *Bcl-xL* (G138A) (G and H), and pPTN290, the vector control (I and J).

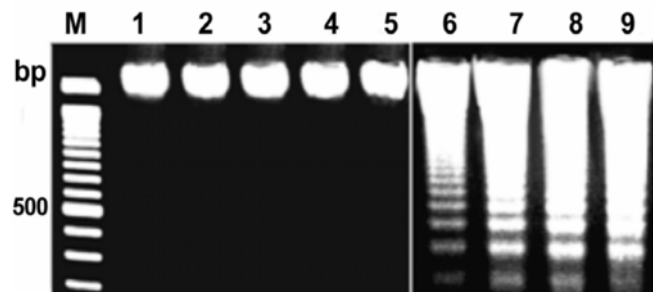


Fig. 7. Inhibition of *Agrobacterium*-induced nuclear DNA fragmentation in root cells of banana. Genomic DNA was isolated after 72 h of exposure to *Agrobacterium* spp. at an optical density at 600 nm of 0.5. Lane 1: untransformed banana roots not exposed to *Agrobacterium* (control); lanes 2, 3, and 4: *Bcl-xL*, *CED-9*, and *Bcl-2* 3' untranslated region transformed root cells, respectively, exposed to *Agrobacterium* spp.; lane 5: *Bcl-xL* (G138A)-transformed roots, not exposed to *Agrobacterium* spp.; lanes 6, 7, 8, and 9: untransformed root cells (cv. Grand Nain), untransformed root cells (cv. Lady finger), pPTN290 (β -glucuronidase)-transformed root cells, and *Bcl-xL* (G138A)-transformed root cells, respectively, exposed to *Agrobacterium* spp.

63x 1.4NA oil PL APO objective lenses under a Leica TCS 4D confocal laser scanning microscope (Leica, Heidelberg, Germany), equipped with Argon/Krypton and UV lasers (ex. splitter DD488/586) and Leica TCS-4D software.

Molecular analysis of transgenics.

Genomic DNA was isolated from plant tissues using cetyltrimethylammonium bromide buffer supplemented with 1.4% (wt/vol) 2-mercaptoethanol and standard chloroform extractions. Gene-specific primers were used to confirm the presence of transgenes and non-T-DNA region primers were used to confirm the absence of *Agrobacterium* spp. contamination in the intracellular spaces of plant tissues. RNA was extracted using a Qiagen RNeasy Plant Mini Kit (Qiagen Inc.) and analyzed using Titan One-Tube RT-PCR Kit (Roche Diagnostics) following the manufacturer's instructions and using gene-specific primers as above. For Southern analysis, genomic DNA extracts were digested with restriction enzyme *SacI* or *EcoRV* that cut only once within the T-DNA. Hybridization and detection of the probe was carried out using the DIG Luminescent Detection kit for Nucleic Acids (Roche Diagnostics).

Agroinfection of transgenic plant roots.

The root tip cells were synchronized by incubating the roots for 15 h in 2.5-mM hydroxyurea-supplemented rooting media, followed by 6 h of recovery. Young, actively growing roots were immersed in 1.5 ml of *Agrobacterium* cell suspension for 30 min at 22°C and co-cultivated as above.

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