Evidence for *Agrobacterium*-Induced Apoptosis in Maize Cells

Geneviève Hansen

Novartis Agribusiness Biotechnology, Inc. (NABRI), 3054 Cornwallis Road, Research Triangle Park, Durham, NC 27709, U.S.A.

Accepted 14 February 2000.

***Agrobacterium* spp. can genetically transform most dicotyledonous plant cells whereas many monocot species are recalcitrant to *Agrobacterium*-mediated transformation. One major obstacle is that co-cultivation of *Agrobacterium* spp. with plant tissues often results in cell death. Report here is that, in maize tissues, this process resembles apoptosis, with characteristic DNA cleavage into oligonucleosomal fragments and morphological changes. Two anti-apoptotic genes from baculovirus, *p35* and *iap*, had the ability to prevent the onset of apoptosis triggered by *Agrobacterium* spp. in maize tissues. *p35* is reported to act as a direct inhibitor of a certain class of proteases (caspase) whereas *iap* may act upstream to prevent their activation. This evidence raises the possibility that caspase-like proteases may also be involved in the apoptotic pathway in plant cells.**

The soil bacterium *Agrobacterium tumefaciens* has evolved a natural plant-transformation mechanism in which a DNA segment, the T-DNA, located on the tumor-inducing (Ti) or root-inducing (Ri) plasmids travels by a conjugation-like process from the bacterium to the plant nucleus and becomes integrated into the plant's chromosomal DNA. *A. tumefaciens* provides an excellent route for introducing foreign DNA into most plants (Hansen and Chilton 1999). In addition, transgenic plants frequently contain a simple integration pattern of the transgene. With the access to a wide array of genes with potential for crop improvement, the interest in optimizing the performance of this system and applying it to all crops has intensified.

A major obstacle in *Agrobacterium*-mediated transformation can be the incompatibility between *A. tumefaciens* and plant cells. In many cases, phytopathogenic bacteria carry *hrp* (hypersensitive reaction and pathogenicity) genes whose products are involved in the elicitation of the hypersensitive response (HR) in resistant plants (Baker et al. 1997). In this interaction, a nonhost plant species recognizes the phytopathogen and induces a rapid defense response (HR) that results in localized necrosis at the site of infection and confines further invasion. Tissue necrosis involving T-DNA genes is ob-

served in certain plant-*Agrobacterium* spp. interactions (Deng et al. 1995) but the symptoms appear more slowly than in a typical HR and necrosis extends beyond the inoculation site. The necrotic response induced by wide-host-range (De Cleene and De Ley 1976) *Agrobacterium* strains on grapevines has been ascribed to cell death at the site of inoculation (Yanofsky et al. 1985). In this case, necrosis is blocked by mutations within the *virC* locus although T-DNA transfer occurs (Yanofsky et al. 1986). It has also been reported that *Agrobacterium* strains with a disarmed vector used in standard transformation protocols could affect some plant tissues from various species, hindering the recovery of transgenic plants. For instance, exposure of grape embryogenic calli (*Vitis vinifera*) to diluted *Agrobacterium* cultures results in tissue necrosis and subsequent death (Perl et al. 1996). This reaction seems to correlate with elevated levels of peroxides and is prevented by the addition of antioxidants such as polyvinylpyrrolidone and dithiothreitol (Perl et al. 1996). In both aspen and poplar, *Agrobacterium*-induced necrosis is likely caused by an accumulation of ammonium and could be reduced with addition of Ca gluconate in the culture medium (de Block 1990). In the case of maize, recalcitrance to transformation by *Agrobacterium* spp. is not clear (Chilton 1993; Smith and Hood 1995). T-DNA transfer can occur and the nuclear localization signals carried by the virulence gene products VirD2 and VirE2 involved in T-DNA transfer and integration are recognized in maize cells (Hansen et al. 1994; Zupan et al. 1996). It was suggested that the recalcitrance may reside in a block of T-DNA integration and in the lack of cell division during wound response (Binns and Thomashow 1988; Narasimhulu et al. 1996).

In all multicellular organisms, cell death can occur by necrosis, a passive process involving disruption of membrane integrity and subsequent swelling that results in lysis, or by programmed cell death (PCD), an active process in which the cell directs its own death (Ellis et al. 1991; Goldstein 1997). PCD has been described as occurring in animal cells to remove unwanted cells during development and maintenance of tissues. There are also many examples of PCD in plants occurring as part of tissue development and in the HR triggered by avirulent pathogens (Jones and Dangl 1996; Levine et al. 1996; Greenberg 1997; Gilchrist 1998; Buckner et al. 1998; Pennell and Lamb 1997). The isolation of spontaneous cell death mutants in Arabidopsis and in maize, in which PCD is activated in the absence of pathogens or stress, suggests that PCD is under genetic control in plants, as reported for vertebrates and invertebrates (Greenberg 1997; Gilchrist 1998).

Corresponding author: Geneviève Hansen; Novartis Agribusiness Biotechnology, Inc. (NABRI), 3054 Cornwallis Road, Research Triangle Park, Durham, NC 27709, U.S.A.; Telephone: 1-919-541-8685; Fax: 1-919-541-8585; E-mail: genevieve.hansen@nabri.novartis.com

PCD proceeds through several stages generally implemented by a mechanism involving de novo gene expression. In this cascade of events, the components of the pathway include regulatory proteins that are conserved among vertebrates and invertebrates and a family of cysteine proteases known as caspases because they cleave specific substrates at Asp residues. In addition, cytochrome c release from the mitochondria has been observed and is involved in the activation of caspases. In the final phase, cells undergo extensive structural changes including cell and nuclear shrinkage, chromatin condensation, DNA fragmentation, and disassembly of the nuclear and cytoplasmic networks (Reed 1997; Raff 1998).

Activation of members of the caspase family has been shown to be both necessary and sufficient to trigger apoptosis in animal cells and is considered the final committing step in the pathway (Raff 1998; Kumar and Colussi 1999; Wolf and Green 1999). Synthetic peptide analogs have been developed that specifically inhibit caspase activity and are useful in blocking the cellular apoptosis program in a variety of systems. It has also been reported that apoptosis can be blocked in vertebrates and in invertebrates by two types of anti-apoptotic proteins encoded by baculovirus: p35 and inhibitors of apoptosis (IAPs) (Teodoro and Branton 1997; Bump et al. 1995; Miller 1999). p35 isolated from *Autographa californica nuclear polyhedrosis virus* (AcMNPV) is a broad-spectrum caspase inhibitor that normally blocks apoptosis in SF-21 cells derived from *Spodoptera frugiperda* (Clem et al. 1991). The second baculovirus gene found to prevent apoptosis, *iap*, was identified by its ability to functionally substitute for p35 in a complementation assay (Crook et al. 1993; Birnbaum et al. 1994; Clem and Miller 1994). Homologues of baculovirus IAPs have also been identified in eukaryotes and more recently in plants (Cahoon and Klein 1999).

A hypersensitive type of response can be observed when maize cells are exposed to *Agrobacterium* spp. and may be part of the incompatibility mechanism. In this report, this response is characterized and is shown to have several aspects indicative of apoptosis or PCD. Furthermore, two anti-apoptotic genes reported to function efficiently in vertebrates and invertebrates were found to prevent the onset of apoptosis triggered by *Agrobacterium* spp. in maize cells.

RESULTS

Sensitivity of maize embryos and embryogenic callus to *Agrobacterium* spp.

Maize immature embryos as well as the derived embryogenic callus lines grown in liquid or in solid medium have been used as target tissues for various transformation procedures (Armstrong 1999; Hansen and Wright 1999). In a typical transformation experiment with *Agrobacterium* spp. as a vector to deliver genes, plant cells suitable for regeneration are co-cultivated with *Agrobacterium* spp., followed by a procedure to select and regenerate transformed cells. Proliferative embryogenic maize cells were thus subjected to inoculation with *Agrobacterium* spp.. Most embryos and embryogenic callus lines as well as suspension cells did not survive the inoculation with the *Agrobacterium tumefaciens* strain LBA4404 at a concentration in the range of 0.2 to 1×10^9 cells per ml (Table 1; Fig. 1A–D). This concentration range and this strain are used in standard transformation experiments (Ishida et al.

1996; Negrotto et al., *in press*). As illustrated in Figure 1, most embryos treated with *Agrobacterium* spp. turned brown and did not develop calli from the scutellum on callus initiation medium. Similarly, embryogenic calli turned brown after a treatment with *Agrobacterium* spp. and did not exhibit proliferating yellow clusters typical of somatic embryos (compare Figure 1B and D). Cell death was observed even after the transfer of the treated embryogenic calli to a cefotaxime-containing medium 5 to 10 min after inoculation. Washing the tissues two or three times with tissue culture medium after inoculation did not prevent the reaction. In control experiments, cefotaxime did not cause maize tissues to die.

Variations can exist among genotypes with respect to performance in tissue culture. The effect of *Agrobacterium* spp.

Table 1. Response of maize suspension cells to co-cultivation with *Agrobacterium* spp.

Scoring ^a	Incubation time with <i>Agrobacterium</i> spp. ^b							
	0.2×10^9 cells per ml				10^9 cells per ml			
	10 min	1 h	24 h	48 h	10 min	1 h	24 h	48 h
2 days	–	+	+	+	+	+	+	++
4 days	+	+	+	++	+	+	++	+++
7 days	+	++	++	+++	+	++	++++	++++
No bacteria	–	–	–	–	–	–	–	–

^a Presence or absence of necrosis was scored 2, 4, and 7 days after inoculation with strain LBA4404.

^b Relative necrosis intensity: –, no necrosis; +, rare formation of necrosis; ++, weak necrosis; +++, strong necrosis; +++++, very strong necrosis.

Fig. 1. Sensitivity of maize embryos and embryogenic callus to *Agrobacterium* spp. Maize immature embryos and callus from the inbred line CG00526 were inoculated with *Agrobacterium tumefaciens* LBA4404 at a density of 1×10^9 cells per ml for 5 min, then plated on cefotaxime-containing medium. Pictures were taken 2 weeks after inoculation. **A**, Nontreated embryo. **B**, Nontreated callus. **C**, *A. tumefaciens*-treated embryo. **D**, *A. tumefaciens*-treated callus. **E**, Callus transgenic for *iap* treated with *A. tumefaciens* 2 weeks after inoculation. Bar = 10 mm.

was, however, observed on maize tissues from three different genotypes—CG00526, CG2717, and HE/89. This result indicated that the effect of *Agrobacterium* spp. on maize tissues appeared to be rather genotype independent.

Cell death mechanism.

To determine the mechanism of cell death induced by *Agrobacterium* spp. in maize tissues, genomic DNA was extracted from suspension cells harvested 24 and 48 h after inoculation with *Agrobacterium* spp. and subjected to electrophoresis. The study of DNA fragmentation was conducted with suspension cells rather than with embryos because this system offered the possibility of isolating DNA from large batches of cells dividing synchronously. Exposure of maize cells to *A. tumefaciens* LBA4404 caused the appearance of discrete bands of DNA after electrophoresis of undigested DNA whereas no DNA laddering was detected in cultures grown in the absence of *A. tumefaciens* LBA4404 (Fig. 2). This pattern is considered an early hallmark of PCD and is associated with the presence of an endogenous nuclease that cleaves chromosomal DNA between individual nucleosomes, producing degraded fragments of approximately 180 bp (Stein and Hansen 1999). Within 24 h after inoculation, the DNA ladder was detectable on agarose gel and the accumulation of fragments dramatically increased 48 h after inoculation. As a control, DNA was extracted from tobacco suspension cultures that were in culture for 24 or 48 h with an *Agrobacterium* sp. As illustrated in Figure 2, no DNA fragmentation was detected on agarose gel. DNA extracted from an *Agrobacterium* sp. cultured in the presence or absence of maize cells showed no degradation, indicating that the observed DNA laddering was derived from the maize genome (data not shown).

Fig. 2. DNA laddering induced by *Agrobacterium* spp. in maize suspension cells. Chromosomal DNA was extracted from (lanes 1, 4, 7) untreated plant suspension cells or from suspension cells inoculated with *Agrobacterium tumefaciens* LBA4404 for (lanes 2, 5, 8) 24 or (lanes 3, 6, 9) 48 h. Approximately 1 to 2 μ g of undigested genomic DNA was run on a 2% agarose gel and stained with ethidium bromide. Lanes 1–3, HE/89 maize cells; lanes 4–6, CG2717 maize cells; lanes 7 and 8, NT1 tobacco cells. Left lane, 123-bp ladder.

No DNA degradation was detected in maize cells pretreated with cycloheximide at a concentration of 40 μ M 30 min prior to the inoculation and washed three times extensively with fresh culture medium. Inoculation of maize cells with *Escherichia coli* or with autoclaved *Agrobacterium* spp. cells contained high molecular weight DNA with no evidence of degradation. It was also observed that suspension maize culture in contact with *Agrobacterium* spp. for 48 h and washed of *Agrobacterium* spp. cells failed to induce cell death of fresh culture. DNA fragmentation was also detected in maize tissues after inoculation with *Agrobacterium* strains cured of the Ti plasmid carrying the virulence functions needed for T-DNA transfer into plant cells. This indicated that the process of genetic transformation by *Agrobacterium* spp. was not required to trigger cell death (data not shown).

Intranucleosomal DNA cleavage can also be examined by an assay that allows for in situ detection of DNA breaks at the single cell level. This method is based on the labeling of the 3' ends of DNA strand breaks. As illustrated in Figure 3B, embryo cells treated with *Agrobacterium* spp. did show some dark precipitates localized in the nuclei whereas no staining could be observed in the control experiment carried out with embryos plated on callus initiation medium (Fig. 3A). Also, maize cells became distorted in shape after exposure to *Agrobacterium* spp. (Fig. 3B). This morphological change was obvious 24 h after inoculation. Interestingly, cytological signs of DNA cleavage were observed in embryos treated with *Agrobacterium* spp. within a few hours of the onset of cell degradation whereas the distortion of maize cells started to be apparent at a later stage.

In several animal models, cytochrome c release from mitochondria into the cytosol is one of the earliest events in apoptosis and is required for the activation of the caspase protease cascade, as well as downstream events including DNA laddering. To track cytochrome c distribution within maize cells, protein extracts were separated into particulate and soluble fractions, containing mitochondrial and cytosolic proteins, respectively, and analyzed by Western blot (immunoblot) with an anti-cytochrome c monoclonal antibody (Stein and Hansen 1999). Maize cells were cultured in the absence or presence of *A. tumefaciens* LBA4404 for 62, 72, or 88 h. The subcellular distribution of cytochrome c in these cultures is shown in Figure 4. In the noninfected cultures, most of the cytochrome c

Fig. 3. Detection of apoptosis in maize embryos inoculated with *Agrobacterium* spp. Cross sections of embryos from genotype CG00526 (A) nontreated and (B) treated with LBA4404 24 h after inoculation. Bar = 20 μ m.

was detected in the particulate fraction, consistent with localization within mitochondria. In contrast, in *Agrobacterium*-infected cultures, cytochrome c was distributed in both the particulate and soluble fractions, reaching a maximum at 88 h. No bands were detected in protein extracted from *Agrobacterium* spp. (data not shown). Concurrently, portions of each culture were simultaneously assayed for the presence of DNA laddering, providing an indication of apoptotic cell death. Cultures infected with *Agrobacterium* spp. displayed DNA laddering that increased over the course of infection, correlating with the level of cytochrome c release observed (data not shown).

The data demonstrating DNA cleavage together with the results on the distribution of cytochrome c indicated that *Agrobacterium* spp. could trigger the process of apoptosis in maize cells.

Testing of cell death suppressor genes in maize tissues.

Two cell death suppressor genes, *p35* and *iap*, reported to function efficiently in vertebrates and in invertebrates were introduced into maize to test whether their expression could reduce or inhibit the effect triggered by *Agrobacterium* spp. on maize tissues. The coding regions of *p35* and *iap* from baculovirus were cloned into a plant expression cassette.

In a first approach, *p35* and *iap* were cloned onto a binary vector and delivered into embryos and embryogenic calli with *Agrobacterium* spp. Following co-cultivation, maize tissues were examined. Tissue survival and proliferation were quantified to evaluate whether the expression of these genes had conferred protection. The results are presented in Table 2. About 25% of embryos treated with *Agrobacterium* spp. harboring either *p35* or *iap* gave rise to embryogenic callus whereas only 3% of embryos inoculated with a control *Agrobacterium* strain exhibited callus initiation. In control, approximately 80% of nontreated embryos gave rise to embryogenic calli.

Fig. 4. Release of cytochrome C in cytosol after inoculation with *Agrobacterium* spp. Maize cells were cultured in the absence (–) or presence (+) of *Agrobacterium* spp. for 62, 72, and 88 h. Cells were fractionated into pellet (P) and soluble (S) fractions and immunoblotted with an anti-cytochrome c antibody.

genic calli. A similar effect was observed with calli inoculated with *Agrobacterium* spp. harboring *p35* or *iap*. These results suggested that *p35* and *iap* were expressed in planta and that their expression reduced tissue browning to some extent but did not completely inhibit the phenomenon. Under the conditions tested, transient expression of these genes was likely required to protect the challenged maize cells. It is also conceivable that not all challenged cells were transformed, or expressed the anti-apoptotic genes.

In a second approach, we tested whether *p35* and *iap* could prevent the *Agrobacterium*-induced apoptosis when stably expressed in maize callus. This assay consisted of delivering *p35* and *iap* genes under the control of a plant-expressible promoter into callus lines with the biolistic device. These constructs were co-bombarded with the *pat* selectable marker gene and transgenic callus lines were recovered on phosphinothricin (PPT) selection. Five transgenic callus lines were recovered from 16 pieces of calli bombarded with the *pat* gene only. With the same parameters, three and five callus lines were generated containing *p35* and *iap*, respectively. All the callus lines also contained the *pat* gene. All independent callus lines were then subjected to inoculation with *Agrobacterium* spp. and the number of lines surviving the treatment was recorded. A typical result of one experiment is illustrated in Table 3. Transgenic callus lines for *iap* and *p35* did not exhibit cell death after inoculation with *Agrobacterium* inoculation whereas control tissues exhibited cell death and failed to proliferate further. An example for *iap* is shown in Figure 1E. These results were reproducible on each of the callus lines.

The protective effect of *p35* and *iap* was also apparent when measured by oligonucleosomal DNA fragment electrophoresis (Fig. 5). DNA was extracted from transgenic callus following inoculation with *Agrobacterium* spp. as shown above. DNA fragmentation was clearly observed in calli transgenic for *pat* alone (Fig. 5, lanes 1–3) but not for *iap* (Fig. 5, lanes 4–6) or *p35* (data not shown). To further correlate expression of the cell death suppressor genes and the absence of cell death after inoculation with *Agrobacterium* spp., the expression of *p35* and *iap* was analyzed by reverse-transcription-polymerase

Table 3. Response to inoculation by *Agrobacterium* spp. of callus transgenic for *p35* and *iap*

Transgenes in transformed tissues	No. of calli resistant to LBA4404 ^a	
	Line 1	Line 2
pUbiAc	1/24 (4%)	1/32 (3%)
p35, pUbiAc	22/25 (88%)	25/28 (89%)
iap, pUbiAc	20/22 (90%)	19/25 (76%)

^a No. of proliferating callus pieces /no. of callus pieces tested (%).

Table 2. Response of maize embryos and calli after inoculation with *Agrobacterium* spp. containing anti-apoptotic genes:

Genes tested	Response of maize target tissues to co-cultivation with <i>Agrobacterium</i> spp.			
	Embryos		Calli	
	Experiment no. 1	Experiment no. 2	Experiment no. 1	Experiment no. 2
No bacteria	52/63 (82%) ^a	25/31 (80%)	76/80 (95%) ^b	72/80 (90%)
LBA4404	2/55 (3%)	3/89 (3%)	8/80 (10%)	6/80 (7.5%)
LBA4404 (<i>p35</i>)	19/75 (25%)	18/74 (24%)	23/80 (29%)	27/80 (33%)
LBA4404 (<i>iap</i>)	22/89 (24%)	21/75 (28%)	25/80 (31%)	30/80 (37%)

^a Number of embryos with callus initiation/number of embryos tested (%).

^b Number of proliferating callus pieces /number of callus pieces tested (%).

chain reaction (RT-PCR). An example is shown in Figure 6. A clear band of the expected size appeared in extracts from *iap* and *p35* transgenic callus, confirming their expression in maize. To exclude contamination of genomic DNA in the RT-PCRs, control reactions were prepared in parallel. PCRs performed with the primers only in the absence of any added DNA did not amplify any fragments. Also, no band was detected if the PCR was conducted on an aliquot of mRNA after DNase treatment, or on an aliquot of reaction in which the reverse transcriptase was omitted.

DISCUSSION

This report shows that the cell death triggered by *Agrobacterium* spp. in maize tissues has many of the features of apoptosis. DNA fragmentation and cytological changes as well as cytochrome c release were shown to occur upon inoculation with *Agrobacterium* spp. Further evidence for *Agrobacterium* spp. inducing DNA fragmentation was obtained by investigating the sensitivity of the process to Zn^{2+} and Ca^{2+} . Addition in the culture of Ca^{2+} increased the intensity of DNA ladders whereas the addition of Zn^{2+} markedly reduced genomic fragmentation (data not shown). These characteristics are fully consistent with the effect of these divalent cations on the endogenous endonucleases responsible for DNA cleavage during apoptosis in animal systems and in some cases in plant cells (Peitsch et al. 1993, Mittler and Lamb 1995). Whether the endonucleases induced by *Agrobacterium* spp. have any functional similarity with the ones induced by other pathogens remains to be determined. The intensity of the ladder was also decreased by addition of cy-

cloheximide to the medium prior to inoculation, evidence that the process requires de novo gene expression.

Two anti-apoptotic genes from baculovirus, *p35* and *iap*, had the ability to prevent the onset of apoptosis triggered by *Agrobacterium* spp. in maize tissues. *p35* is reported to act as a direct inhibitor of caspases whereas *iap* may act upstream to prevent activation of caspases. Caspases drive most if not all of the terminal effector events of apoptosis in vertebrates and in invertebrates and are responsible, either directly or indirectly, for the cleavage of cellular proteins. Such proteins includes nuclear proteins such as poly(ADP-ribose) polymerase, DNA-dependent protein kinase, and lamins, as well as actin (Nagata 1997). The finding that these genes could affect the reaction of maize cells to an apoptotic stimulus strongly indicates that plants, like animals, may have caspase-like proteases regulating apoptosis. Interestingly, a caspase-like proteolytic activity was recently reported in tobacco plants undergoing an HR triggered by an infection with *Tobacco mosaic virus* (del Pozo and Lam 1998).

An important event in animal PCD is the release of cytochrome c from mitochondria into the cytosol. Cytochrome c release is responsible for triggering caspase activity and initiating the final degradation phase of the cell death program. In this report, cytochrome c release was observed in maize cells following exposure to *Agrobacterium* spp. This release correlated with DNA fragmentation and cell death. In plants, overexpression of Bax-1, involved in the release of cytochrome c in animal cells, was found to induce an HR in tobacco (Lacomme and Cruz 1999). These observations point to the possibility of a similar mechanism in both animal and plant cells.

Because *p35* and *iap* interfere with the apoptosis phenomenon, one could expect that their expression would affect plant growth by inhibiting the PCD normally occurring during plant development. For instance, overexpression could lead to inappropriate survival of cells normally signaled to die by the pathway inhibited by *p35* and *iap*. Although the size of the

Fig. 5. Inhibition of DNA fragmentation induced by *Agrobacterium* spp. in maize calli transgenic for the anti-apoptotic gene. DNA was isolated from maize calli after inoculation with *Agrobacterium* spp. and analyzed by electrophoresis on a 2% agarose gel. Lane 1W, a 123-bp DNA ladder. DNA from callus transgenic for the selectable marker only (lane 1) nontreated, or treated with *Agrobacterium* spp. for (lane 2) 24 and (lane 3) 48 h. DNA from callus transgenic for *iap* (lane 4) nontreated, or treated with *Agrobacterium* spp. for (lane 5) 24 and (lane 6) 48 h.

Fig. 6. Expression analysis of *iap* and *p35* by reverse transcription-polymerase chain reaction (RT-PCR) in transgenic maize calli. Control PCRs were performed (lane 1) with the primers only and no DNA added, (lane 2) on an aliquot of mRNA extract after DNase treatment, (lane 3) on an aliquot of the reaction in which the reverse transcriptase was omitted, and (lane 6) on cDNA from nontransgenic calli. RT-PCR performed with (lane 4) 0.5 μ g of cDNA and (lane 5) 1 μ g of cDNA.

experiment doesn't allow a definitive conclusion to be drawn, the phenotype of the transgenic plants was comparable to the control plants with a normal senescence pattern. This observation could be explained by (i) the presence of other factors involved in the activation of the proteases and/or the factor(s) were not totally shut off, or (ii) these particular events did not show high expression in all tissues. It is interesting to note that maize embryos inoculated with the strain containing the cell death suppressor genes often showed mixed areas of living and necrotic cells. This observation could be explained by the fact that (i) only cells transformed by the T-DNA carrying the anti-apoptotic gene survived and that the number of these cells may be small compared with the total mass of cells challenged by *Agrobacterium* spp.; (ii) individual transformed cells did not necessarily transmit the signal to neighboring cells; and (iii) the cell death suppressor genes rescued doomed cells very efficiently but the level of transient expression was not sufficient to confer full protection against *Agrobacterium* spp.

Previous work has shown that *Agrobacterium* spp. can transform maize even though it is generally not its natural host (Chilton 1993; Smith and Hood 1995). For instance, transgenic maize plants were recovered after inoculation of immature embryos from the inbred A188 (Ishida et al. 1996; Negrotto et al., *in press*). This report shows, however, that *Agrobacterium* spp. can trigger cell death on maize tissues of three different maize genotypes, one of them containing A188 as part of its genetic background. There may be genetic differences in susceptibility to *Agrobacterium*-induced PCD, as observed in other host-pathogen interactions. In addition, tissue type (embryo versus callus) may contribute to these differences in susceptibility (Lupotto et al. 1999).

Despite numerous observations that *Agrobacterium* spp. can induce necrosis in plant tissues from various species, there has been no previous evidence that this form of cell death proceeds via apoptosis. A molecular understanding of this mechanism may allow one to minimize *Agrobacterium*-induced apoptosis and develop more efficient transformation protocols. A search for nucleosomal ladders in plant species refractory to transformation by *Agrobacterium* spp. will show whether apoptosis is a common outcome of incompatible *Agrobacterium*/plant interactions.

MATERIALS AND METHODS

Maize lines.

Friable embryogenic callus of the maize line HE/89 that is a derivative of A188 (Mórocz et al. 1990) was grown in a modified liquid N6 medium (Chu et al. 1975) amended with 500 mg of Bacto tryptone per liter, 30 g of sucrose per liter, and 0.5 mg of 2,4-dichlorophenoxy acetic acid (2,4-D) per liter (0.5N63S). The suspension maize proprietary cell line CG2717 was grown in N6 liquid medium supplemented with 30 g of sucrose per liter and 2 mg of 2,4-D per liter (2N63S). The *Nicotiana tabacum* cell line NT-1 was grown in Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 2 mg of 2,4-D per liter and sucrose (30 g/liter) (MS3S). Suspension cultures were maintained in liquid medium on a rotary shaker (120 rpm; G10 gyrotory shaker, New Brunswick Scientific, Edison, NJ) and were subcultured every 7 days.

Embryos and nonfriable type I embryogenic callus of the maize inbred CG00526, a proprietary elite line developed from a Lancaster-type population, were used in some of the experiments. Type I calli were initiated from immature embryos (0.8 to 2.5 mm) that were aseptically excised 12 to 15 days after pollination and plated scutellum up on Duncan medium supplemented with 20 g of sucrose per liter and 5 mg of Chloramben per liter (5CD2S) (Kozziel et al. 1993). The calli that were initiated from embryos were subcultured approximately every 2 weeks on Duncan medium supplemented with 0.5 mg of 2,4-D per liter (0.5D2S).

Bacterial growth and *Agrobacterium* strains.

Agrobacterium strains were grown for 3 days on YP medium (5 g of yeast extract per liter, 10 g of peptone per liter, 5 g of NaCl per liter, 15 g of agar per liter, pH 6.8) supplemented with 50 mg of spectinomycin per liter and 10 mg of tetracycline per liter when needed. *A. tumefaciens* LBA4404 (pAL4404) is a derivative of ACH5 (Ooms et al. 1982). pAL4404 is a disarmed helper plasmid. A136 and C58NT1 are cured of the Ti plasmid. Bacteria were centrifuged and resuspended in the appropriate plant medium at a density ranging from 0.2×10^9 to 5×10^9 cells per ml. For the inoculation of CG2717, HE/89, CG00526, and NT-1 plant cells, bacteria were resuspended in 2N63S, 0.5N63S, 0.5D2S, or MS3S liquid medium, respectively.

Vectors for biolistic transformation.

Vectors used to transform maize by the biolistic device were all derivatives of pUC. pUbiAc contains a plant-expressible gene encoding for the phosphinothricin acetyl transferase (PAT) conferring resistance to phosphinothricin (Wohllleben et al. 1988) driven by the maize ubiquitin promoter (Christensen et al. 1992). The coding regions of *p35* and *iap* were cloned under control of the maize metallothionein-like gene promoter (MTL) (DeFramond et al. 1991) to engineer pNOV122 and pNOV 123, respectively. The *p35* coding region was cloned as a *Pst*I-*Eco*RI fragment from pHSP35VI⁺ (Clem and Miller 1994). The *iap* coding region was cloned as an *Sal*I-*Spe*I fragment from pHSCpIAPVI⁺ (Clem and Miller 1994).

Vectors for transformation by *Agrobacterium* spp.

p35 and *iap* from pNOV122 and pNOV123 were cloned between the border sequences into the binary vector pSB11 (Ishida et al. 1996). These vectors were then introduced into LBA4404 (pAL4404, pSB1) by electroporation.

Transformation by bombardment.

Plasmid DNA was precipitated onto 0.3 to 1 μ m of gold microcarrier as described in the DuPont Biolistic manual (du Pont de Nemours, Wilmington, DE). Two micrograms of pNOV122 or pNOV123 and 2 μ g of pUbiAc were used per 50 μ l of microcarrier. Maize calli (eight pieces per plate) were bombarded with the PDS-1000/He biolistic device. Tissues were placed on the shelf 8 cm below the stopping screen shelf and a $10 \times 10 \mu$ m stainless steel screen was used with rupture disks of 650 lb/in² value. After bombardment, tissues were selected on 100 mg of PPT per liter. Six to 8 weeks later, tissues were transferred onto 40 mg of PPT per liter. Transgenic tissues were either used directly for inoculation experiments or transferred onto regeneration medium (Murashige and

Skoog medium containing 3% sucrose, 0.25 mg of ancymitol per liter and 5 mg of kinetin per liter) with 16 h of light per day. Putative transgenic plants were analyzed by PCR and further by RT-PCR (see below).

Inoculation of maize embryos and transgenic callus lines with *Agrobacterium* spp.

Embryos were inoculated with *Agrobacterium* spp. at a density of 0.2 to 1×10^9 cells per ml for 5 min and then co-cultured on the callus initiation medium for 3 days. Tissues were then transferred on the same medium supplemented with cefotaxime (250 mg/liter). Transgenic callus lines obtained as described above were inoculated with *Agrobacterium* spp. by soaking them in bacterial suspension for 5 to 10 min. After inoculation, maize tissues were co-cultured in the dark at 25°C on 0.5D2S. Tissues were transferred on the same medium supplemented with cefotaxime (250 mg/liter) 2 or 3 days after inoculation. All experiments were conducted three times.

For all experiments, the effect of *Agrobacterium* spp. on callus lines and on embryos was quantified by measuring the weight 2 weeks after co-culture. At this stage, about 90% of control embryos have produced calli from the scutellum of the embryo and control callus lines have proliferated and doubled in size.

Inoculation of suspension cells with *Agrobacterium* spp.

Friable embryogenic callus of CG2717, HE/89, and NT-1 maintained in 50 ml of liquid medium was incubated with *Agrobacterium* spp. for 10 min or 1h, 24, or 48 h prior to transfer to cefotaxime-containing medium. Fifty microliters of a bacterial suspension (0.2×10^9 or 2×10^9 cells per ml) was added to 10 ml of tissue culture medium containing 1 ml of packed cell volume. If the culture was to be incubated longer than 2 days, bacteria were washed off and plant cells were re-suspended in the same medium supplemented with cefotaxime (250 mg/liter).

In one experiment, maize suspension cells were inoculated with *Agrobacterium* spp. as described above and the co-cultivation lasted 48 h. Maize cells were then centrifuged and washed three times with liquid culture medium containing cefotaxime (250 mg/liter). The cells were resuspended in 10 ml of fresh medium and placed in a petri dish. The cells were covered either with a filter paper 3MM (Whatman International, Springfield Mill, Kent, UK) or with a 200×200 stainless steel mesh with 200 openings per linear 2.54 cm horizontally and vertically (McMaster-Carr, New Brunswick, NJ). About 1 ml of fresh maize cells was placed on the top of the filter or mesh. Alternatively, treated maize cells were embedded in 0.5% low melting agar (Seaplaque; FMC, Rockland, ME) and poured in a 250-ml flask. About 1 ml of fresh maize cells grown in 50 ml of liquid medium supplemented with cefotaxim was added to the flask.

Analysis of transgenic material.

Genomic DNA was extracted from 100 mg of callus with a DNA extraction kit (Isoquick; Orca Research, Bothwell, WA) and resuspended in 20 µl of water. One microliter was used for PCR. PCRs were performed in a Perkin-Elmer Cetus thermal cycler (Norwalk, CT). Each 25-µl reaction contained $1 \times$ PCR buffer, 0.5 U of AmpliTaq DNA polymerase (Roche, Branchburg, NJ), 200 µM concentrations of each dNTP, and

0.2 µM concentrations of each primer. To detect the presence of the *pat* gene in tissues, PCRs were performed with the primers PAT1 and PAT2. The primer set used to detect *iap* was P and I. The primer set P and 35 was used to detect *p35* in transgenic callus lines. All reactions were conducted at an annealing temperature of 55°C for 35 cycles.

PAT1: 5'-TGTCTCCGAGAGGAGACC -3';
PAT2: 5'- CCAACATCATGCCATCCACC-3';
MTL (P): 5'-AGGTGTCCATGGTGTCTCAAG-3';
iap (I): 5'-ACAATCGAACCGCACACGTC-3';
p35 (35): 5'-CCAGGTAGCAGTCGTTGTGT-3'

***p35* and *iap* RT-PCR.**

Total RNA was extracted from transgenic callus with Tripure Isolation Reagent (Boehringer Mannheim, Mannheim, Germany). RNA was treated with RNase-free DNase, and 0.5 µg was utilized for cDNA synthesis with Advantage RT-PCR (Clontech, Palo Alto, CA) and oligo-dT primer. After the second-strand synthesis, one-twentieth of the reaction was used as a template for PCR with Taq DNA polymerase. *p35* primers used for RT-PCR were as follows: 5'-GGTCCTATACGAAGCGTTAC and 5'-CCACGTAGCAGTCGTTGTGT, which amplifies 300 bp of transcript. *iap* primers were 5'-CATGTCTGACTTGCGATTGG and 5'-ACAATCGAACCGCACACGTC, which amplifies 248 bp of transcript. All reactions were conducted at an annealing temperature of 55°C for 30 cycles.

DNA extraction for DNA fragmentation detection.

Cells were removed at various times after inoculation with *Agrobacterium* spp. and chromosomal DNA was isolated as described by Hall et al. (1991). DNA was extracted from 0.5, 0.5, and 1 g of cells from NT1, CG2717, and HE/89, respectively. Approximately 1 to 2 µg of undigested genomic DNA sample was run on a 2% agarose gel containing 0.1 µg of ethidium bromide per ml.

In situ cytological detection of DNA fragmentation.

The assay involves end-labeling the DNA fragments by the Klenow enzyme with biotylated nucleotides that are conjugated to a detectable marker such as streptavidin fluorescein. Increased DNA fragmentation leads to a staining of the nucleus. Embryos were soaked in 3.7% formaldehyde solution and processed as described by the manufacturer (Trevigen, Gaithersburg, MD). To detect genomic DNA cleavage in situ, fixed embryos were treated with the TACS-1 kit (Trevigen). Fixed cross sections were first treated with proteinase K for 5 min at room temperature and then dipped for 5 min into 2% hydrogen peroxide to remove endogenous peroxidase. After a brief wash with Klenow buffer, tissues were incubated for 1 h at 37°C with the Klenow enzyme. A dark, insoluble precipitate indicative of apoptosis formed in individual cells and could be detected microscopically (Cullivier 1996).

Cytochrome c distribution.

Maize suspension cells (CG2717) were incubated in the presence or absence of *A. tumefaciens* LBA4404 (approximately 4×10^8 cells per ml at $T = 0$) for 62, 72, and 88 h, and cytochrome c distribution was analyzed as described previously (Stein and Hansen 1999). A portion of each culture was fractionated into particulate (P) containing mitochondria and soluble (S) fractions containing the cytosol. Fractions

were immunoblotted with the mouse monoclonal antibody clone 7H8.2C12 at a concentration of 2 µg/ml (PharMingen, San Diego, CA) as follows: cells were washed and suspended in mitochondria isolation buffer (50 mM Tris pH 7.5; 10 mM MgCl₂; 1 mM CaCl₂; 1 mM EDTA; 0.25 M sucrose; 1 mM AEBSF; 2 µg of pepstatin A per ml (wt/vol); 10 µg of leupeptin per ml (wt/vol); 10 µg of aprotinin per ml (wt/vol); 0.1% (vol/vol) Antifoam 204) and homogenized with a polytron. The homogenate was filtered through Miracloth (Calbiochem, San Diego, CA) and centrifuged at 1,000 × g for 10 min. The resulting supernatant was centrifuged at 10,000 × g for 10 min and the particulate fraction residing in the pellet was resuspended in mitochondria isolation buffer. The supernatant was further centrifuged at 100,000 × g for 30 min to obtain a soluble fraction in the supernatant. Protein concentration was assayed by the Bradford method (Bio-Rad, Hercules, CA) and 5 µg of protein was loaded per lane on a 4–20% polyacrylamide gel (Mark 12; Novex, Frankfurt, Germany). The gel was electrophoresed in the presence of sodium dodecyl sulfate and dithiothreitol, and electroblotted onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA). The blot was developed with a chemiluminescent substrate (Supersignal; Pierce, Rockford, IL) according to the manufacturer's instructions.

ACKNOWLEDGMENTS

I would like to thank Lois K. Miller for providing the clones pHSP35VI⁺ and pHSCpIAPVI⁺, Joshua Stein for conducting the experiment on cytochrome c localization, and Allan Wenck for critical reading of the manuscript.

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