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(57) **Abrégé/Abstract:**

An improved method of Agrobacterium transformation of plants, particularly Gramineae, is provided, utilizing conditions capable of inhibiting Agrobacterium-induced necrosis.



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<p>(54) Title: PLANT TRANSFORMATION METHODS</p> <p>(57) Abstract</p> <p>An improved method of <i>Agrobacterium</i> transformation of plants, particularly Gramineae, is provided, utilizing conditions capable of inhibiting <i>Agrobacterium</i>-induced necrosis.</p>		

PLANT TRANSFORMATION METHODS

This invention relates to a method of transforming plants using *Agrobacterium* by carrying out the transformation in the presence of an agent which inhibits *Agrobacterium*-induced necrosis and/or using a strain of *Agrobacterium* which does not induce necrosis.

The first method and still one of the most widely used methods for the introduction of foreign genetic material into plants exploits the natural transformation system of *Agrobacterium spp.* using recombinant Ti (tumor-inducing) or Ri (root-inducing) plasmids wherein the T-DNA comprises a gene of interest. The gene of interest is incorporated into the plant's genetic material by the same mechanism as is used in the natural system to incorporate the oncogenes or opine biosynthesis genes.

While *Agrobacterium* transformation works well for plants which are naturally infected and transformed by *Agrobacterium* in the wild to form tumors and/or hairy roots, it does not work well for other plants. For example, members of the grass family (Gramineae) such as maize, are not known to form tumors upon exposure to *Agrobacterium* and have generally proved to be extremely recalcitrant to *Agrobacterium* transformation. Ishida, et al. Nature Biotechnology (1996) 14: 745-750 have reported transformation of embryos from a particular maize line (A188) and its hybrids using *Agrobacterium* having "super-binary" vectors, but the reproduceability of these results and the applicability of this system to other maize lines and or using *Agrobacterium* having other vectors is unclear. There have also been reports of *Agrobacterium* transformation of maize using apical meristem as target tissue, but the low efficiency of apical meristem as a target tissue compared to, e.g., embryogenic callus, make this approach less than optimal. Even some dicotyledonous plants such as grapes, soybeans or pepper which are susceptible to *Agrobacterium* infection and tumor formation in the wild have nevertheless proved difficult to transform in the laboratory because the preferred target tissues for transformation and regeneration seem to respond poorly to *Agrobacterium* exposure. The lack of understanding as to the mechanisms of resistance to *Agrobacterium* infection and tumor formation has proved an obstacle to devising a solution to or even to properly evaluating the problem of *Agrobacterium* transformation in plants recalcitrant to *Agrobacterium* transformation, such as Gramineae.

It has now surprisingly been discovered that *Agrobacterium* is capable of inducing apoptotic necrosis in plant cells, particularly of the family Gramineae, e.g., maize. Methods

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of selecting and generating *Agrobacterium* which are less competent to induce necrosis have been discovered, as have methods of inhibiting the necrosis response in cells to be transformed.

The necrosis seen in Gramineae cell culture upon exposure to *Agrobacterium* is a programmed cell death by which the cell, apparently through a signal transduction mechanism, directs its own death. It is distinct from passive cell death, i.e., necrosis involving disruption of membrane integrity and subsequent swelling that results in lysis, for example as is seen upon exposure to various toxins. Cells undergoing programmed cell death show characteristic morphological changes and DNA fragmentation, which, collectively, define the process of apoptosis and are implemented by a mechanism involving *de novo* gene expression. The stereotypic characteristics of apoptosis described in animal cells are shrinkage, loss of cell-to-cell contact in organized tissues, condensation of the nucleus and chromatin, fragmentation of DNA (DNA "laddering") and nuclear and membrane blebbing. We have shown that cocultivation of *Agrobacterium* with maize or wheat tissues results in a process closely analogous to apoptosis in animal cells, wherein cell death is characterized by DNA cleavage into oligonucleosomal fragments and defined morphological changes. In maize cells exposed to *Agrobacterium*, Ca^{2+} increases the intensity of DNA fragmentation whereas Zn^{2+} has the opposite effect, which parallels the effect of these divalent cations on endogenous endonucleases responsible for DNA cleavage during apoptosis in animal cells. This fragmentation is also decreased by addition of cycloheximide, which is evidence that the process requires *de novo* gene expression. Programmed cell death in response to exposure to *Agrobacterium* has not previously been reported.

It has further been discovered that this *Agrobacterium*-induced necrosis (AIN) observed in Gramineae can be inhibited by the use of AIN-inhibiting agents, either chemical compounds such as silver nitrate, or AIN-inhibiting nucleotide sequences stably integrated or transiently operative in the cell to be transformed. It has also been discovered by screening collections of *Agrobacterium* that *Agrobacterium* strains vary in their ability to induce necrosis, so that strains can be selected which are suitable for transforming recalcitrant plants such as Gramineae.

Accordingly, the several embodiments of the invention include the following:

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1. A method of transforming a plant cell with a gene of interest, comprising exposing said plant cell to *Agrobacterium* under conditions which inhibit AIN such as the presence of an AIN-inhibiting agent or heat shock treatment, wherein said *Agrobacterium* comprises one or more plasmids (vectors) comprising one or more genes of interest.

Thus, in one aspect, the present invention provides a method of making a transgenic plant cell, comprising: (a) transforming a plant cell with a gene of interest by co-cultivating said plant cell with *Agrobacterium* under conditions which inhibit *Agrobacterium* Induced Necrosis (AIN), wherein said *Agrobacterium* comprises a vector comprising said gene of interest and wherein said conditions are selected from the group consisting of: (i) co-cultivating said plant cell with *Agrobacterium* after heat-shock treatment of said plant cell; and (ii) co-cultivating said plant cell with *Agrobacterium*, wherein said plant cell has been transformed with a nucleotide sequence which encodes an mRNA or protein from a gene selected from the group consisting of: baculovirus p35, baculovirus inhibitors of apoptosis (iap), and dad-1, in operative linkage with a promoter; and to produce a transgenic plant cell having a transgene comprising said gene of interest; (b) selecting said transgenic plant cell.

In another aspect, the present invention provides a method of making a fertile transgenic plant comprising: (a) transforming plant tissue by co-cultivating said plant tissue with *Agrobacterium* under conditions which inhibit *Agrobacterium* Induced Necrosis (AIN), wherein said *Agrobacterium* comprises a vector comprising a gene of interest and said conditions are selected from the group consisting of: (i) co-cultivating said plant tissue with *Agrobacterium* after heat-shock treatment of said plant

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tissue; and (ii) co-cultivating said plant tissue with *Agrobacterium*, wherein cells of said plant tissue have been transformed with a nucleotide sequence which encodes an mRNA or protein from a gene selected from the group consisting of: baculovirus p35, baculovirus inhibitors of apoptosis (iap), and dad-1, in operative linkage with a promoter; to produce a transgenic plant tissue, the cells of which have a transgene comprising said gene of interest; and (b) regenerating said transgenic plant tissue to produce said fertile transgenic plant.

In another aspect, the present invention provides a transformed plant cell produced according to the method described above.

1.1. In one embodiment of the foregoing method, the AIN-inhibiting agent is a chemical inhibitor. The chemical inhibitor is preferably a compound selected from the group consisting of ethylene inhibitors (e.g., 2,5-norbornadiene, norbornene, silver thiosulfate, and silver nitrate), ethylene synthesis inhibitors (e.g., aminoethoxyvinylglycine (AVG), cobalt salts, acetyl salicylic acid, or salicylic acid), gibberelin antagonists (e.g., abscisic acid (ABA)) and phosphatase inhibitors (e.g., okadaic acid). Most preferably, the chemical inhibitor is an ethylene inhibitor, preferably silver nitrate. Proteins and peptides can act as chemical inhibitors as well. Examples are naturally occurring proteins such as DAD-1, the baculovirus inhibitors of apoptosis (IAPs), baculovirus p35, or synthetic peptide analogs of caspases capable of triggering apoptosis. A chemical inhibitor is suitably present in an effective concentration, e.g., for silver nitrate in a concentration of from 0.1 to 20 mg/l, preferably 1 to 10 mg/l.

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1.2.1. In an alternative embodiment of this method, the AIN-inhibiting agent is a nucleotide sequence. The AIN-inhibiting nucleotide sequence may inhibit AIN directly or by encoding an AIN-inhibiting mRNA coding for an AIN-inhibiting protein. For example, it may be an antisense oligonucleotide or a gene encoding antisense mRNA, which is antisense to a gene encoding a necrosis associated enzyme (e.g., protease, kinase, or phosphatase) or regulatory protein. Alternatively, it may comprise the coding region of a gene capable of inhibiting apoptosis under control of a promoter capable of expression in plants, e.g., a coding region of a mammalian bcl-1 gene under control of a promoter capable of expression in plants, a coding region of an apoptosis-inhibiting gene from a baculovirus such as p35 or pIAP, or a gene capable of suppressing disease response in plants, e.g., nahG, dad-1, or mlo. An AIN-inhibiting nucleotide sequence expressing an AIN-inhibiting protein may optionally be adapted for expression in the host plant by making a synthetic nucleotide sequence encoding the same protein but using codons which are preferred by the host plant and avoiding nucleotide sequences, e.g.,

polyadenylation signals or splice sites within the coding region, which may affect optimal expression in the host plant, e.g., analogously to the methods described in US 5,380,831 or US 5,610,042.

1.2.2 The AIN-inhibiting nucleotide sequence may be stably incorporated into the genome of the plant cell to be transformed or may be only transiently present and operable, e.g., at or around the time the cell is exposed to the *Agrobacterium*. Transient expression can be obtained, e.g., using an agroinfection system, wherein the T-DNA carries two gemini viruses in tandem such that a viral replicon that carries the AIN-inhibiting nucleotide sequence may replicate in the cell. In this system, the virus typically will not integrate into the plant cell genome but will replicate to a high copy number and provide a high level of transient expression. Cells thus primed to be resistant to necrosis can then be transformed using *Agrobacterium* having Ti plasmids comprising the gene of interest, which will be incorporated into the genome, while the virus is diluted through regeneration and will not be transmitted to the seed. Thus the progeny and descendants of the infected plant cell are stably transformed with the gene of interest but not with the AIN-inhibiting nucleotide sequence. Transient expression may alternatively be obtained by introducing short AIN-inhibiting oligonucleotide sequences into the plant cell, e.g., antisense sequences.

1.3. In a further alternative embodiment AIN is reduced or inhibited by heat-shock treatment of the plant tissue to be transformed prior to cocultivation with *Agrobacterium*. The heat shock treatment is performed at 40-50°C, preferably at 42-48°C, 45°C being a preferred temperature for maize. The treatment lasts for 2-10 minutes and preferably 4-8 minutes.

2. A method of making a fertile, transgenic plant comprising transforming plant tissue by the method of 1 above, and regenerating the tissue thus transformed. Preferably, the tissue is selected from immature embryos or embryogenic calli, e.g., maize type I callus. Where the tissue is immature embryo, the method preferably further comprises placing the embryos on callus initiation medium either before or after exposure to *Agrobacterium* and regenerating the plant from the embryogenic callus thus obtained. Preferably, the gene of interest (or one of them) is a selectable or scorable marker, permitting selection or identification of transformed cells, transformed tissue and/or regenerated transformed plants.

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3. The use of an AIN-inhibiting agent, e.g., a chemical inhibitor of AIN (for example an ethylene inhibitor, e.g., silver nitrate) or an AIN-inhibiting nucleotide sequence (for example one encoding for an AIN-inhibiting mRNA or protein) in a method of *Agrobacterium* transformation of a plant or tissue or cell thereof or a method of making a fertile transgenic plant, e.g., a method according to 1 or 2 above.
4. A transgenic plant produced by the method of 2 above, or seed or progeny thereof
5. A plant, plant tissue or plant cell comprising a nucleotide sequence of heterologous origin which inhibits AIN, e.g., a synthetic nucleotide sequence or a nucleotide sequence derived from the genome of a different species of organism.
6. A culture of plant cells or tissues, e.g., for use in transformation of Gramineae, comprising
 - a) a chemical inhibitor of AIN,
 - b) an *Agrobacterium* comprising a plasmid comprising a gene of interest, and
 - c) water and essential salts.

The chemical inhibitor is suitably present in a necrosis -inhibiting concentration. The chemical inhibitor is preferably a compound selected from the group consisting of ethylene inhibitors (e.g., 2,5- norbornadiene, norbornene, silver thiosulfate, and silver nitrate), ethylene synthesis inhibitors (e.g., aminoethoxyvinylglycine (AVG), cobalt salts, acetyl salicylic acid, or salicylic acid), gibberelin antagonists (e.g., abscisic acid (ABA)) and phosphatase inhibitors (e.g., okadaic acid). Most preferably, the chemical inhibitor is silver nitrate, e.g., in a concentration of from 0.1 to 20 mg/l, preferably 1 to 10 mg/l. The optimal composition and concentration of essential salts are as known in the art and may vary somewhat depending on the species, type, and stage of development of the cells or tissue, but are generally such that the ion and nutrient concentrations of the medium (e.g. concentrations of nitrates, potassium ions, phosphates, calcium ions, magnesium ions, sodium ions, and chlorine ions) are maintained at levels which are well tolerated by the plant cells and by the *Agrobacterium*. The culture medium may optionally further comprise suitable nutrients (e.g., sugars, for example sucrose), vitamins, amino acids, hormones and other components (e.g., 2, 4-D) as known in the art of plant cell or tissue culture. The plant

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is preferably of the family Gramineae, e.g., maize. The cells or tissues preferably comprise embryogenic callus, e.g., Type I or Type II callus.

7. A method of transforming a totipotent cell of a plant of the family Gramineae, comprising exposing a population of totipotent cells or tissue comprising totipotent cells to *Agrobacterium* comprising one or more plasmids comprising one or more genes of interest, wherein the *Agrobacterium* is of a strain which does not induce significant levels of necrosis in said population at an exposure duration and concentration sufficient to achieve transformation of said cell. The tissue is preferably other than apical meristem, e.g., preferably undifferentiated tissue, e.g., plated zygotic embryos or embryogenic callus, preferably embryogenic callus during initiation phase (e.g., up to 28 days, preferably up to 14 days from plating on initiation medium), or serially propagatable embryogenic callus, e.g., Type I or Type II callus.

8. A method for determining the suitability of an *Agrobacterium* strain for use in the transformation of a regenerable cell of a plant of the family Gramineae comprising exposing a population of said regenerable cells of the plant to the *Agrobacterium* strain and observing or determining the amount of necrosis in said cell population.

9. An *Agrobacterium* strain which has been genetically modified to reduce or eliminate expression of the *Agrobacterium* necrosis factor or a derivative of such a modified strain. *Agrobacterium* necrosis factor is the heat labile factor observed in concentrated supernatant capable of inducing necrosis, e.g., programmed cell death, in maize embryos.

The incompatibility between *Agrobacterium* and maize cells likely involves some genetic components. The present invention describes three genes from *Agrobacterium* involved in the cell death response of maize tissues. They are identified by screening a BAC library of *Agrobacterium*. 3 independent BAC are found to elicit cell death and show homology with *xyIA-xyIB*, *virB1* and *acvB*. It is possible that the genes reported are only a subset of the genes responsible for the incompatibility of *Agrobacterium* with maize tissues. The plant cell used in the methods of the invention is preferably from a plant species or variety which does not produce galls or hairy roots upon *Agrobacterium* transformation. Preferably the plant species is a member of the grass family (Gramineae), most preferably maize or wheat, especially maize. The plant cell is preferably a totipotent cell, i.e., a cell which is capable of

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regenerating itself into a plant, preferably a fertile plant. Totipotent cells are present, e.g., in immature embryos and embryogenic calli. The target tissue is preferably other than apical meristem. More preferably, the target tissue comprises undifferentiated tissue, e.g., plated zygotic embryos or embryogenic callus. The embryogenic callus may be associated with the embryo during the callus initiation phase (e.g., the period up to 28 days, preferably up to 14 days from plating of the immature embryo on initiation medium), or may be serially propagatable embryogenic callus, e.g., Type I or Type II callus. Maize Type I callus is a preferred tissue comprising totipotent cells for use in the present invention.

The *Agrobacterium* is preferably selected from *A. tumefaciens* and *A. rhizogenes*.

Preferably the *Agrobacterium* strain is an *A. tumefaciens* strain, most preferably a nopaline-utilizing strain. When the *Agrobacterium* strain is an *A. rhizogenes* strain, it is preferably an agropine- or mannopine-utilizing strain. Most preferably, the *Agrobacterium* is an *Agrobacterium* which does not induce necrosis in Gramineae, e.g., an *Agrobacterium* selected from *A. tumefaciens* strains A and B. *Agrobacterium* strains A and B have been deposited with the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, Maryland 20852 / USA, under ATCC Designation numbers 55964 and 55965 respectively on May 2, 1997, pursuant to the Budapest Treaty.

The *gene of interest* is preferably a gene for herbicide resistance, disease resistance, or insect resistance, or is a selectable or scorable marker, and comprises a plant-operable promoter, a coding region, and a 3' terminator region. Herbicide resistance genes include the AHAS gene for resistance to imidazolinone or sulfonyl urea herbicides, the pat or bar gene for resistance to bialaphos or glufosinate, the EPSP synthase gene for resistance to glyphosate, etc. Disease resistance genes include genes for antibiotic synthetic enzymes, e.g., for pyrolnitrin synthetic enzymes, plant derived resistance genes, and the like. Insect resistance genes include genes for insecticidal proteins from *Bacillus thuringiensis*.

Selectable markers include herbicide resistance genes and antibiotic (e.g., hygromycin or kanamycin) resistance genes, as well as positive selectable markers, such as the gene for mannose phosphate isomerase. Scorable markers include genes for readily assayable enzymes such as the gus gene and the cat gene. The plasmid may comprise more than one gene of interest and/or the *Agrobacterium* may comprise different plasmids having different genes of interest.

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The term *Agrobacterium*-induced necrosis (AIN) as used herein refers to any *Agrobacterium*-mediated plant cell death, but particularly includes *Agrobacterium* induced programmed cell death.

EXAMPLES

Example 1 - Mechanism of action of necrosis in maize exposed to *Agrobacterium*

a. Demonstration of necrosis of maize but not tobacco in presence of *Agrobacterium*

Maize embryogenic calli and embryos have proven to be particularly difficult tissues for transformation by *Agrobacterium*. The following experiments employ maize inbred lines (*Zea mays* L.) HE/89 and a line here designated as Elite 1. Friable embryogenic callus of the maize line HE/89 was provided by Gunter Donn. HE/89 is described in Mórocz, S., Donn, G., Németh, J. and Dudits, D. (1990) *Theor. Appl. Genet.* 80:721-726). Elite 1 is a Novartis proprietary elite line related to B73. Suspension culture of Elite 1 is initiated from friable embryogenic callus selected from immature embryos. The HE/89 line is grown in a modified liquid N6 medium amended with 500 mg/l Bacto tryptone, 30 g/l sucrose and 0.5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) (N6M). The Elite 1 line is grown in N6 liquid medium (Chu et al., 1975) supplemented with 30 g/l sucrose and 2 mg/l 2,4-D (2N63S). The *Nicotiana tabacum* cell line NT-1 grown in Murashige and Skoog medium supplemented with 2 mg/l of 2,4-D and sucrose (30g/l) (MS3S) is used as an *Agrobacterium*-susceptible control. Suspension cultures are maintained in liquid medium on a rotary shaker (120 rpm) and are subcultured every 7 days.

Bacteria are grown in YP medium (5 g/L yeast extract, 10 g/l peptone, 5 g/l NaCl, pH 6.8) for 24 hr at 28°C. Bacteria are centrifuged and resuspended in the appropriate plant medium. For the inoculation of Elite 1, HE/89 and NT-1 plant cells, bacteria are resuspended in 2N63SM, N6M and MS3S liquid medium respectively.

Soon after inoculation of maize embryogenic callus, many of the cells turn necrotic (Table 1). Necrosis is observed even after the transfer of the treated embryogenic calli to a bacteriae free-, cefotaxime-containing medium. Cocultivation of maize cells with very diluted cultures of *Agrobacterium* for a very short time is sufficient to induce necrotic response or inhibition of growth of the maize inbred Elite 1. For HE/89 embryogenic calli, a short time of cocultivation reduces necrotic change to some extent, but does not completely prevent necrosis. In control experiments, cefotaxime does not cause the maize tissue to become

necrotic. Thus, it appears that the necrotic effect is a consequence of the interaction between *Agrobacterium* and maize calli. As control, NT1 tobacco cells are also inoculated with *Agrobacterium* and no necrogenesis is observed.

Table 1: Cocultivation of plant suspension cells with LBA4404

	HE/89			Elite		
	2 days	4 days	7 days	2 days	4 days	7 days
No <i>Agrobacterium</i>	-	-	-	-	-	-
OD=0.2						
10 min	-	-	+	-	+	+
1 hr	-	-	+	+	+	++
24 hrs	-	+	+	+	+	++
48 hrs	+	+	++	+	++	+++
OD=1						
10 min	-	+	+	+	+	+
1 hr	-	+	+	+	+	++
24 hrs	+	+	++	+	++	++++
48 hrs	+	+	++	++	+++	++++

Friable embryogenic callus of HE/89 maize and of a proprietary elite inbred maize maintained in liquid medium are incubated with *Agrobacterium* for 10 min, 1 hr, 24 hr or 48 hrs prior to transfer to cefotaxim-containing medium. 50 µl of bacteria at an optical density indicated (OD=600 nm) is added to 10ml of tissue culture medium containing 1 ml of packed cell volume. If the culture is to be incubated longer than two days, bacteria are washed off and plant cells are resuspended in the same medium supplemented with cefotaxime (250 mg/l). The presence or absence of necrosis is scored 2, 4 and 7 days after inoculation and the relative intensity of necrosis is expressed as follow: +, rare formation of necrosis; ++, weak necrosis; +++, strong necrosis; +++++, very strong necrosis.

b. Evidence for programmed cell death

In order to determine the mechanism of cell death induced by *Agrobacterium* in maize tissues, genomic DNA is extracted from plant suspension cells harvested 24 or 48 hours after inoculation with *Agrobacterium*. Exposure to *Agrobacterium* causes DNA fragmentation in both maize lines, with a pattern characteristic of internucleosomal fragmentation, which is considered an early hallmark of programmed cell death. Control maize cells and tobacco cells, maintained in culture medium alone, do not show DNA fragmentation under these conditions. Inoculation of maize cells with *E.coli* or with autoclaved *Agrobacterium* cells does not cause DNA fragmentation. Furthermore, maize cells undergoing programmed cell death could not induce cell death of fresh maize embryogenic tissues.

Further evidence for *Agrobacterium* inducing DNA fragmentation is obtained by investigating the sensitivity of the process to Zn^{2+} and Ca^{2+} . In the cell line HE/89, Ca^{2+} increased the intensity of DNA ladders whereas Zn^{2+} markedly reduced genomic fragmentation. These characteristics are fully consistent with the effect of these divalent cations on the endogenous endonucleases responsible for DNA cleavage during apoptosis in animal system. The intensity of the ladder also is decreased by addition of cycloheximide, evidence that the process requires *de novo* gene expression. These results indicate that the contact of *Agrobacterium* with maize cells accounts for the cell death of maize tissues. Fragmentation of DNA during apoptosis is also detected *in situ* by reagents that react with the exposed hydroxyls on the nucleosome units. 12 day old maize embryos from a proprietary elite line of Lancaster parentage are inoculated with LBA4404 and plated on DG4 medium supplemented with chloramben. Koziel, M.G., & al. 1993. *Bio/Technology* 11: 194-200. Apoptotic cells are detected by in situ staining with TACS-1* kit (Trevigen), which involves end labeling the DNA fragments by the klenow enzyme using dNTP conjugated to a detectable marker, giving a dark insoluble precipitate indicative of genomic fragmentation. Cullivier, O., Pirianov, G., Kleuser, B., Vanek, P.G., Coso, O.A., Gutking, J.S. and Spiegel, S. (1996) *Nature* 381, 800-803. Embryos are soaked in 3.7% formaldehyde solution and processed as described by the manufacturer. To detect genomic DNA cleavage *in situ*, fixed embryos are treated with the TACS-1 kit. Fixed cross-sections are 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 then incubated for 1 hr at 37°C with the

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klenow enzyme that can incorporate exogenous modified deoxynucleotides to the 5' termini of the cleaved fragments. A dark insoluble precipitate indicative of apoptosis that appears in the individual cell is detectable with a microscope.

Agrobacterium-treated cells become darkened and distorted in shape after 48-hr exposure. Control embryos, maintained in culture medium alone or inoculated with *E.coli*, do not show any precipitate under these conditions. It is also found that not all maize tissues react similarly to an *Agrobacterium* infection: shoots remain intact whereas embryos turn necrotic. DNA fragmentation is also observed with *Agrobacterium* strains cured of the pathogenic plasmid (Strains A136 and LBA4402), suggesting that the factor inducing programmed cell death is unrelated to the plasmid. Programmed cell death is also observed when maize embryos are incubated for 20 minutes with supernatant concentrated 20-fold from a cell death inducing *Agrobacterium* (strain LBA 4404), but not when embryos are contacted with supernatant prepared in the same way from *E. coli*. Programmed cell death also does not occur if the concentrated *Agrobacterium* supernatant is heated for 20 minutes at 100° C prior to incubation.

Maize embryogenic calli often contain areas of cells still apparently alive and other areas which are necrotic. The fact that only limited areas die, even in situations in which cells outside the responding area are exposed simultaneously to the stimulus suggests that temporal or permanent diversity exists in elements controlling the response of individual cells to different stimuli in organized tissues. The number of cells in an organized tissue undergoing programmed cell death may be small compared with the total mass and the process may be asynchronous. The induction of PCD in one or a few cells in a given area or tissue may trigger the rapid death of surrounding cells as observed in animal cells, and the latter cells may not show the programmed cell death phenotype but die nonetheless.

Example 2: Inhibition of *Agrobacterium*-induced programmed cell death

Preparation of tissues for inoculation with *Agrobacterium*:

Immature embryos (1.2 to 2.2 mm in length) are aseptically excised 14-15 days after pollination from surface-sterilized, greenhouse-grown ears and plated scutellum up on callus initiation medium, 2DG4 + 5 mg/l chloramben (2DG4-5Chl). 2DG4 medium is Duncan's medium modified to contain 20 mg/l sucrose.

Embryos with callus response:

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Immature embryos are sterilized as described above and plated on callus initiation medium (2DG4-5Chl) and cultured therein for 1 to 7 days. The resulting tissue is used as samples for inoculation.

Young type I callus:

Embryos plated on callus initiation medium (2DG4 + 5 Chloramben) for 7 to 20 days give rise to callus. These calli are typical type I calli for the maize inbred used, which are compact and relatively well organized clusters of cells. Resultant embryogenic callus are cut out of embryos and transferred to callus maintenance medium, 2DG4 + 0.5 mg/l (2,4-dichlorophenoxy) acetic acid (2,4-D) and used as samples for inoculation.

Type I callus:

Type I callus obtained by the method described above can be maintained on the maintenance medium (2DG4 + 2,4 D 0.5 mg/l) and subcultured every 2 weeks.

Agrobacterium:

Strain *A. tumefaciens* LBA4404 (pAL4404, pSB1) is used in these experiments. pAL4404 is a disarmed helper plasmid. pSB1 is a wide host range plasmid that contains a region of homology to pGIGUP and a 15.2 kb KpnI fragment from the virulence region of pTiBo542 (Ishida et al., 1996; High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*, Nature Biotechnology 14, 745-750). The introduction of the plasmid pGIGUP by electroporation into LBA4404(pAL4404, pSB1) results in a cointegration of pGIGUP and pSB1. The T-DNA of this plasmid contains a plant expressible PAT gene driven by the ubiquitin promoter to provide resistance to phosphinothricin and a gene for GUS with an intron in the N-terminal codon of the coding sequence driven by the Gelvin promoter. This intron-GUS gene expresses GUS activity in the plant cell but not in *Agrobacterium*.

Bacterial growth:

Agrobacterium is grown for 3 days on YP medium (5g/l yeast extract, 10g/l peptone, 5g/l NaCl, 15 g/l agar, pH 6.8) supplemented with 50 mg/l spectinomycin and 10 mg/l tetracycline. Bacteria are collected with a loop and suspended in N6 liquid medium at a density ranging from 10^9 to 5×10^9 cells/ml. *Agrobacterium* cells can also be collected from an overnight culture in YP medium and resuspended in N6 liquid medium.

Co-cultivation in the presence or absence of silver nitrate:

Maize tissues prepared as described above are inoculated with *Agrobacterium*. Maize tissues are soaked in the bacterial suspension for 5-10 min and then plated on medium with or without silver nitrate (1 to 10 mg/l). Tissues are also inoculated with a 5 µl drop 10^9 to 5×10^9 cells/ml of an *Agrobacterium* suspension placed on the top of the tissue. After inoculation, maize tissues are cultured in the dark at 25°C with or without silver nitrate in the medium (1 to 10 mg/l). 2 or 3 days after inoculation, tissues are transferred on the same medium supplemented with cefotaxime (250 mg/l) with or without silver nitrate.

Results:

Sensitivity of maize embryos and maize embryogenic calli to *Agrobacterium*.

Embryogenic calli can develop from maize embryos of when plated on callus initiation medium. The cell line developed from embryos remains highly embryogenic when maintained on 2,4-D containing medium. The sensitivity of this embryogenic calli and embryos from a maize inbred line to *Agrobacterium* LBA4404 is assessed. A marked browning of the tissue is observed following cocultivation of *Agrobacterium* and maize tissues. This reaction is also observed with LBA4402 that is cured of its pathogenic plasmid. Control embryos and embryogenic calli that are cocultivated with *Escherichia coli* and subjected to the same procedures do not exhibit cell death or significant browning. Thus, it appears that the cell death is a consequence of the interaction between maize cells and *Agrobacterium*. It appears that embryos are more sensitive to *Agrobacterium* than embryogenic callus. A concentration of 10^9 cells/ml is enough to induce cell death. Furthermore, a short exposure (5 min) is sufficient to induce this reaction.

The effect of adding silver nitrate to the co-cultivation medium and to the callus initiation medium is determined. Addition of silver nitrate allows recovery of healthy tissues (Tables 2 and 3). About 50 % of the embryos plated on medium containing silver nitrate are able to produce embryogenic calli. The optimal effect is obtained with embryos plated on callus initiation medium for at least 2 days prior inoculation with *Agrobacterium*. The combination of these two factors is found to drastically prevent necrosis of maize embryogenic calli following *Agrobacterium* co-cultivation.

The reduced browning of the embryogenic calli correlates with callus initiation and survival of the tissue after *Agrobacterium* inoculation. About 80% of control embryos not treated with *Agrobacterium* give rise to embryogenic calli.

The effect of silver nitrate is also observed with type I calli inoculated with *Agrobacterium* (Table 3). It appears that a young line is more resistant to *Agrobacterium* inoculation and a pretreatment with silver nitrate improves the recovery of healthy tissues.

In order to verify that the presence of the silver nitrate during co-cultivation does not reduce *Agrobacterium* virulence, tobacco leaf discs are inoculated with LBA4404(GIGUP) at a concentration of 10^9 cells/ml. No significant decrease in GUS activity is observed in tobacco leaves.

Table 2: Inoculation of maize embryos with LBA4404

Embryos and embryos with embryogenic callus responses are inoculated with *Agrobacterium* LBA4404 (GIGUP) (10^9 cells/ml). The basic medium is 2DG4+5Chloramben (2DG4). All treatments are done on about 80 to 100 embryos and repeated twice. Callus initiation is scored two weeks after inoculation.

Pretreatment (time)	Co-inoculation medium	Transfer	Score
no pretreatment	2DG4	2DG4 +Cef	1 %
no pretreatment	2DG4	2DG4 + AgNO3 +Cef	30 %
no pretreatment	2DG4 + AgNO3	2DG4 + AgNO3 +Cef	55 %
2DG4 + AgNO3 (1 day)	2DG4 + AgNO3	2DG4 + AgNO3 +Cef	43 %
2DG4 + AgNO3(4 days)	2DG4 + AgNO3	2DG4 + AgNO3 +Cef	27 %
2DG4 (6 days)	2DG4 + AgNO3	2DG4 + AgNO3 +Cef	65 %
2DG4 (6 days) + 1 day on 2DG4 + AgNO3	2DG4 + AgNO3	2DG4 + AgNO3 +Cef	88 %

Table 3: Inoculation of maize type I callus

Type I calli are inoculated with *Agrobacterium* LBA4404 (GIGUP) (10^9 cells/ml). The basic medium used in these experiments is 2DG4+ 0.5 2,4D (2DG4) with or without silver nitrate (AgNO3). All experiments are done on about 150 pieces of type I callus. Survival of tissues is scored 2 weeks after inoculation.

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Type I callus	pretreatment	Co-inoculation	Transfer	Score
1/2 month old	2DG4	2DG4+ AgNO ₃	2DG4+ AgNO ₃ + Cef	75 %
	2DG4+ AgNO ₃	2DG4+ AgNO ₃	2DG4+ AgNO ₃ + Cef	95 %
	2DG4	2DG4	2DG4+ Cef	22%
10 month-old	2DG4	2DG4+ AgNO ₃	2DG4+ AgNO ₃ + Cef	55 %
	2DG4+ AgNO ₃	2DG4+ AgNO ₃	2DG4+ AgNO ₃ + Cef	66 %
	2DG4	2DG4	2DG4+ Cef	12%

Example 3: *Transformation by Agrobacterium of Immature Zygotic Embryos and Isolation of Transformed Callus with the Use of Phosphinothricin, Hygromycin or Mannose as a selection agent.*

Immature embryos are obtained approximately 10 to 14 days after self-pollination. The immature zygotic embryos are divided among different plates containing medium capable of inducing and supporting embryogenic callus formation at about 25 immature embryos per plate.

The immature embryos are inoculated either on the plate or in liquid as indicated in example 2 with *Agrobacterium* having a Ti plasmid comprising a selectable marker gene. Through a series of experiments, optimized conditions are developed for immature embryos. In one optimized condition, the immature embryos are plated on callus initiation medium containing silver nitrate (10 mg/l) either prior or immediately after inoculation with *Agrobacterium*.

Approximately 25 immature embryos are placed onto each plate. 16 to 72 hours after inoculation, immature embryos are transferred to callus initiation medium with silver nitrate and cefotaxim. Selection of transformed cells is carried out as follows:

a. PPT resistance marker: Transformation is carried out using an *Agrobacterium* strain harboring a plasmid with a gene coding for resistance to phosphinothricin on the T-DNA region. Transformed cells are selected *in vitro* by application of phosphinothricin at a concentration of 3 mg/L 2 to 20 days after inoculation and maintained for a total of 2-12 weeks. The embryogenic callus so obtained is regenerated in the presence or absence of phosphinothricin on standard medium of regeneration. All plants are tested by the chlorophenol red (CR) test for resistance to PPT. This assay utilizes a pH sensitive indicator dye to show which cells are growing in the presence of PPT. Cells that grow produce a pH change in the media and turn the indicator Chlorophenol Red yellow (from

red). Plants expressing the resistance gene to PPT are easily identified in this test. Plants positive by the CR test are assayed by PCR for the presence of the PAT gene. Plants which are positive for PCR test are analyzed by Southern blot.

b. Hygromycin resistance marker: Transformation is carried out using an *Agrobacterium* strain harboring a plasmid with a gene (*hpt*, hygromycin B phosphotransferase) coding for resistance to hygromycin on the T-DNA region. Transformed cells are selected using hygromycin at a concentration of 3 mg/L 2 to 20 days after inoculation and maintained for a total of 2-12 weeks. The embryogenic callus so obtained is regenerated in the presence or absence of the selectable agent on standard medium of regeneration. All plants are tested for resistance to hygromycin. Plants expressing the resistance gene to hygromycin are easily identified in this test. Plants positive by this test are assayed by PCR for the presence of the *hpt* gene. Plants which are positive by PCR test are analyzed by Southern blot.

c. Positive selection with mannose: Transformation is carried out using an *Agrobacterium* strain harboring a plasmid with a gene (mannose phosphate isomerase) coding for tolerance to mannose on the T-DNA region, mannose is used to select transformed cells *in vitro*. This selection can be applied as low as 1 g/L 2 to 20 days after inoculation and maintained for a total of 2-12 weeks. The embryogenic callus so obtained can be regenerated in the presence or absence of mannose on standard medium of regeneration. All plants are tested by the chlorophenol red (CR) test for tolerance to mannose. This assay utilizes a pH sensitive indicator dye to show which cells are growing in the presence of mannose. Cells that grow produce a pH change in the media and turn the indicator Chlorophenol Red yellow from red. Plants expressing the tolerance to mannose are easily identified in this test. Plants positive by the CR test are assayed by PCR for the presence of the mannose gene. Plants which are positive for PCR test are analyzed by Southern blot.

Example 4: *Transformation by Agrobacterium of Callus Derived from Immature Zygotic Embryos and Isolation of Transformed callus with the use of phosphinothricin, hygromycin and mannose as selectable agents.*

Type I callus is obtained from immature zygotic embryos using standard culture techniques. Approximately 25 pieces of type I callus are placed on maintenance medium containing silver nitrate either prior or after inoculation with *Agrobacterium*. The inoculation may be performed as described in example 2. Approximately 16-72 hours after inoculation the callus is transferred to standard culture medium containing silver nitrate and cefotaxim. The

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selection can be applied immediately after transfer on this medium or 1 to 20 days after. The callus is then subcultured on selection for approximately 2 to 12 weeks, after which surviving and growing callus is transferred to standard regeneration medium for the production of plants.

Selection is carried out as in the preceding example for cells transformed with genes for phosphinothricin resistance, hygromycin resistance, or mannose phosphate isomerase.

Example 5: *Transformation of Type I callus of maize by inoculation with Agrobacterium and Isolation of Transformed Callus with the Use of Phosphinothricin, Hygromycin and Mannose as Selectable Agents.*

Callus is derived from plating immature embryos of elite genotype. Cultures are subcultured bimonthly on maintenance medium (2DG4 + 0.5mg/l 2,4-D) and cell clumps taken 2-3 days after subculture are placed on 2DG4 medium. After or prior inoculation with *Agrobacterium*, cell clumps are plated on 2DG4 medium containing silver nitrate. Inoculation with *Agrobacterium* may be performed as described in example 2. After 16 to 72 hours incubation, the callus is transferred to fresh maintenance medium containing cefotaxim and silver nitrate. The callus is subcultured on selection for a total approximately 2-12 weeks with the selectable agent, after which surviving and growing callus is transferred to standard regeneration medium for the production of plants. Selection is carried out as in the preceding example for cells transformed with genes for phosphinothricin resistance, hygromycin resistance, or mannose phosphate isomerase.

Example 6: *Heat shock treatment prevents apoptosis triggered by Agrobacterium*
Immature embryos:

Maize inbred lines are grown in the greenhouse. Immature embryos (0.8 to 2.2 mm in length) are aseptically excised from surface-sterilized ears between 8 and 15 days after pollination, depending upon environmental factors. Embryos are plated scutellum up on callus initiation medium. For most of the inbred lines, the embryos are plated on LS medium (Linsmaier and Skoog, *Physiol. Plant.* 18: 100-127, 1965). Embryos of CG00526 are plated on 2DG4 + 5 mg/l chloramben (2DG4-5Chl). 2DG4 medium is Duncan's medium modified to contain 20 mg/l sucrose (Koziel et al., *Bio/Technology* 11: 194-200, 1993).

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Type I callus:

Immature embryos are sterilized as described above and plated on callus initiation medium (2DG4-5Chl) and cultured therein for 1 to 7 days. Embryos plated on callus initiation medium (2DG4 + 5 Chl) for 7 to 20 days give rise to callus. These calli are typical type I calli. Type I calli were compact clusters of relatively well organized cells. Resultant embryogenic callus is cut from the embryos and transferred to callus maintenance medium, 2DG4 + 0.5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) and used for inoculation. Type I callus obtained by the method described above can be maintained on the maintenance medium (2DG4 + 2,4 D 0.5 mg/l) and subcultured approximately every 2 weeks.

Agrobacterium:

The strain used is *A. tumefaciens* LBA4404 (pAL4404, pSB1). pAL4404 is a disarmed helper plasmid (Ooms et al, 7: 15-29, 1982). pSB1 is a wide host range plasmid containing a region of homology to pGIGUP and a 15.2 kb KpnI fragment from the virulence region of pTiBo542 (Ishida et al, Nature Biotechnology 14: 745-750, 1996). The introduction of the plasmid pGIGUP by electroporation into LBA4404(pAL4404, pSB1) resulted in a cointegration of pGIGUP and pSB1. pGIGUP contains a plant expressible phosphinothricin acetyl transferase (PAT) gene driven by the maize ubiquitin promoter to provide resistance to phosphinothricin (Christensen et al, Plant Mol. Biol. 18: 675-689, 1992). It also contains a gene for β -glucuronidase expression (GUS) with an intron in the N-terminal codon of the coding sequence driven by a chimeric promoter derived from the octopine and mannopine synthase genes (a trimer of the octopine synthase promoter upstream activating sequence with a domain of the mannopine synthase promoter, Ni et al, Plant J., 7: 661-676, 1995). This intron-GUS gene expresses GUS activity in the plant cell but not in *Agrobacterium*. *Agrobacterium* is grown for 3 days on YP medium (5g/l yeast extract, 10g/l peptone, 5g/l NaCl, 15 g/l agar, pH 6.8) supplemented with 50 mg/l spectinomycin and 10 mg/l tetracycline. Bacteria are collected with a loop and suspended in N6 liquid medium at a density ranging from 10^9 to 5×10^9 cells/ml. *Agrobacterium* can alternatively be collected from an overnight culture in YP medium and resuspended in N6 liquid medium. It can also be pre-induced for 4 to 6 hrs in *Agrobacterium* Induction Medium (AIM; K_2HPO_4 , 10.5 g; KH_2PO_4 , 4.5 g; $(NH_4)_2SO_4$, 1.0 g; NaCitrate. $2H_2O$, 0.5 g; $MgSO_4 \cdot H_2O$ (1 M), 1.0 ml; Glucose, 2.0 g; Glycerol, 5.0 ml; MES (10 mM); Acetosyringone (50 - 100 μ M); pH 5.6).

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Heat shock treatment of tissues:

Before cocultivation, maize tissues are placed in an Eppendorf* tube in N6 liquid medium and incubated for 4 min at 45°C in a water bath. The medium is then replaced by an *Agrobacterium* suspension prepared as described above. After 5 min at room temperature, tissues are plated on appropriate solid medium. 3 days after co-inoculation, tissues are either stained with X-Glu to detect GUS activity or plated on medium with cefotaxime (250 mg/l). Maize tissues are then examined to detect callus response indicating the percentage of tissues surviving *Agrobacterium* inoculation.

Cocultivation:

Maize tissues prepared as described above are inoculated with *Agrobacterium*. They are soaked in the bacterial suspension for 5-10 min and then plated on solid medium. After inoculation, maize tissues were cultured in the dark at 25°C. 2 or 3 days after inoculation, tissues are transferred onto the same medium supplemented with cefotaxime (250 mg/l).

In situ detection of DNA fragmentation:

Embryos are soaked in a 3.7% formaldehyde solution and processed as described by the manufacturer (Trevigen). To detect genomic DNA cleavage *in situ*, fixed embryos are treated with the TACS-1 kit (Cullivier et al., *Nature* 381: 800-803, 1996). Fixed cross-sections are 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 are incubated for 1 hr at 37°C with the klenow enzyme that can incorporate exogenous modified deoxynucleotides to the 5' termini of the cleaved fragments. A dark insoluble precipitate indicative of apoptosis that appears in the individual cell is detectable with a microscope.

Results:

-- A major browning phenomenon was observed following cocultivation of *Agrobacterium* and maize tissues and a concentration of 10^9 cells/ml could induce cell death. Embryos appeared to be more sensitive to *Agrobacterium* than embryogenic callus.

-- Embryos and type I callus are heat shocked and then inoculated with *Agrobacterium* at a concentration of 10^9 cells/ml. Following three days of co-cultivation and culture for a week, tissues are examined. The level of protection conferred by the heat shock pretreatment is quantified by counting the number of tissues that survive the inoculation.

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All embryos inoculated with *Agrobacterium* after a heat shock pretreatment show callus initiation whereas no callus emerges from embryos that are not heat shocked (Table 4).

Table 4:

Embryos are inoculated with 10^9 cells/ml *Agrobacterium* LBA4404 (pGIGUP) and then plated on callus initiation medium. The basic medium is 2DG4+5Chl (2DG4). All treatments are done on about 80 to 100 embryos and repeated twice. Callus initiation is scored two weeks after inoculation.

Pretreatment	Embryos with callus response / Embryos inoculated
Control	137/150 (91%)
No Heat Shock + Agro	3/150 (2%)
Heat Shock + Agro	112/150 (75%)

The same beneficial effect of the heat shock treatment is observed for callus (Table 5).

Table 5:

Type I callus is inoculated with 10^9 cells/ml *Agrobacterium* LBA4404 (pGIGUP). The basic medium used is 2DG4+ 0.5 2,4D (2DG4). All experiments are performed on approximately 1000 pieces of Type I callus. Survival of tissues is scored 2 weeks after inoculation.

Pretreatment	Survival Score
3 month-old Type I callus line	
Agrobacterium	0/1000
Agro + heat shock	≈1000/1000
10 month-old Type I callus line	
Agrobacterium	0/1000
Agro + heat shock	≈1000/1000

The protection observed after heat shock pretreatment appears to be associated with heat shock because major heat shock proteins can be amplified by RT-PCR.

-- No DNA fragmentation is detected in embryos subjected to a heat shock pretreatment.

-- Embryos from various maize lines are inoculated with *Agrobacterium* strain LBA4404 (GIGUP) induced in AIM solution with or without a heat-shock pretreatment. After three days of co-cultivation, embryos are stained for GUS activity. Results are presented in Table 6. It appears that a heat shock pretreatment has a beneficial effect on transient expression.

Table 6:

Pedigree	Treatment	GUS pos. % of total*	GUS Rating**
0F502	-	15	0.3
	HS	74	1.8
FNU007	-	90	2.0
	HS	96	3.4
HAF031	-	0	0.0
	HS	67	0.9
2N217A	-	13	0.1
	HS	46	0.4
1NJ20	-	5	0.1
	HS	38	0.4
JEF091	-	59	1.2
	HS	81	1.4

- : No heat shock.

HS: heat shock.

*GUS percentages are based on a sample size of approximately 50 immature embryos.

**GUS rating is based on a 0 - 5 scale (0 = no GUS stain, 1 = 1-5 spots/embryo, 2 = 5-15 spots, 3 = 15-30 spots, 4 = ~25% of the embryo surface staining for GUS and ,5 = ~50% of the embryo surface staining for GUS.

Example 7: *Agrobacterium* Transformation of Wheat

Immature zygotic embryos (0.75mm to 1.25mm) are excised and plated on MS-based medium with 3mg/l 2,4-D, 300mg/l glutamine, 150 mg/l asparagine and 3% sucrose (3MS3S) for 0, 3, 4, 5, 6, 7, 8 or 21 days before inoculation with *Agrobacterium*. By 21 days, the explants produce an embryogenic mass referred to as 3-week callus.

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Cocultivation:

Wheat tissues are inoculated with the *Agrobacteria* described in Example 6. Wheat tissues are soaked in the bacterial suspension for 5-10 min and then plated on solid medium. After inoculation, wheat tissues are cultured in the dark at 25°C. 2 or 3 days after inoculation, tissues are transferred onto the same medium supplemented with cefotaxime (250 mg/l). The explants are inoculated without being given a pre-treatment or they are heat shocked at 42-48°C for 4-8 minutes. The heat shock treatment is performed prior to inoculation. Embryos can be heat-shocked in 3MS inf or AIM with 100mM AS liquid whereas calli are heat-shocked "dry".

Inoculation:

Explants are inoculated in either eppendorf tubes or on plates. When inoculating in tubes, 1ml of the *Agrobacterium* solution is pipetted into the tube, finger-vortexed or shaken and allowed to sit for 5-15 minutes. The *Agrobacterium* solution and explant material is then poured onto a plate of 3MS3S with 100mM AS and the liquid is removed with a disposable narrow-tipped transfer pipet. When inoculation is done on plates the *Agrobacterium* is pipetted directly onto the explants and removed 5-15 minutes later. Embryos are arranged so that the embryonic axes are in contact with the medium.

Callus initiation, selection and regeneration:

Embryos are grown on callus induction medium (3MS3S) with antibiotic for 3 weeks. The embryogenic calli are dissected and placed on MS3S (no 2,4-D) with 5mg/l GA₃ and 1mg/l NAA with the selection agent and antibiotic for 2 weeks then removed to MS3S with antibiotic and a higher concentration of selection agent for approximately 4 weeks. The plantlets are then transferred to Magenta boxes with ½ MS salts and 0.5mg/l NAA while keeping the concentration of the selection agent the same as the last step-up. For calli, the selection and regeneration system is the same except that the calli are grown only for a maximum of 6 weeks from embryo plating, that is, 3 week calli are inoculated, co-cultivated and grown for a maximum of 3 more weeks before selection and regeneration starts.

Results:

-- DNA fragmentation is studied in embryos inoculated with *Agrobacterium* as described in Example 6. No DNA fragmentation is detected in embryos subjected to a heat shock pretreatment.

-- Heat shock pretreatment prior to *Agrobacterium* inoculation can prevent the onset of apoptosis.

Example 8: Suppression of *Agrobacterium*-induced apoptosis in maize cells by *p35* and *iap*

Vectors for biolistic transformation:

Vectors used to transform maize by the biolistic device are all derivatives of pUC. pUbiPAT contains a plant expressible *bar* gene encoding for the phosphinothricin acetyl transferase (PAT) driven by the maize ubiquitin promoter (Christensen et al., 1992) to provide resistance to phosphinothricin (PPT). The coding region of *p35*, *iap* and *dad-1* are cloned under control of the maize metallothionein-like gene promoter (MTL) (de Framond, 1991). *p35* and *iap* are provided by Lois Miller (University of Georgia, Athens, Georgia). The *p35* PstI-EcoRI fragment encompassing the coding region is cut out of pHSP35VI* (Clem and Miller, 1994), cloned into the corresponding sites of pBluescript* (Stratagene, La Jolla, California) and then cloned as a PstI-Asp718 fragment into the corresponding sites of pMTL. pMTL contains the MTL promoter and the CaMV35S terminator. The Sall-SpeI fragment encompassing the coding region of *iap* from pHSCpIAPVI* (Clem and Miller, 1994) is cloned into XhoI-SpeI sites of pMTL.

The coding sequence of *dad-1* was cloned as a Sall-XbaI from the Arabidopsis cDNA clone 12T7 (University of Michigan).

Vectors for *Agrobacterium* transformation:

p35, *iap* and *dad-1* coding regions driven by the MTL promoter are cloned between the border sequences into the superbinary vector pSB11.

The β -glucuronidase gene (GUS) with an intron in the N-terminal codon of the coding sequence is driven by a chimeric promoter derived from the octopine and mannopine synthase genes (mas; trimer of the octopine synthase promoter upstream activating sequence with a domain of the mannopine synthase promoter; Ni et al., 1995). Mas-GUS expressing GUS activity in the plant cell but not in *Agrobacterium* is cloned into pSB11 to yield pMasGUS.

These vectors are then introduced into LBA4404 (pAL4404, pSB1) by electroporation with 0.2 cm Bio-Rad* cuvettes at a field strength of 2 kV/cm, resistors of 600 Ohms and a capacitance of 25 μ F. pSB1 is a wide host range plasmid that contains a region of homology to pSB11 and a 15.2 kb KpnI fragment from the virulence region of pTiBo542

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(Ishida et al., 1996). The introduction of the plasmid pSB11 by electroporation into LBA4404(pAL4404, pSB1) results in a cointegration of pSB11 and pSB1.

Inoculation of maize embryos with *Agrobacterium*:

Immature embryos (0.8 to 2.5 mm) are aseptically excised 12 to 15 days after pollination and plated scutellum up on callus initiation medium (2DG4 + 5 Chloramben; Duncan et al., 1985). Embryos are inoculated with *Agrobacterium* at a density of 10^9 cells/ml for 5 min and then cultured on the callus initiation medium for 3 days. Tissues are then transferred on the same medium supplemented with cefotaxime (250 mg/l). Survival of tissues is scored 2 weeks after inoculation.

Initiation of type I callus:

Embryos plated on callus initiation medium for 7 to 20 days give rise to callus. These calli are typical type I calli, which are compact clusters of relatively well organized cells (Suttie et al., 1994). Resultant embryogenic callus is cut out of embryos and transferred to callus maintenance medium, 2DG4 + 0.5 mg/l (2,4-dichlorophenoxy) acetic acid (2,4-D). Type I callus obtained by the method described above can be maintained on the maintenance medium (2DG4 + 2,4 D 0.5 mg/l) and subcultured every 2 weeks approximately. Type I callus is used for inoculation experiments with *Agrobacterium* and for transformation by the biolistic device.

Transformation experiments:

Plasmid DNA is precipitated onto 0.3 to 1 μ m gold microcarrier as described in the DuPont* Biolistic manual. 2 μ g of the anti-apoptotic gene and 2 μ g of pUbiPAT are used per 50 μ l of microcarrier. 16 pieces of type I callus per plate are bombarded using the PDS-1000/He biolistic device (DuPont). Tissues are placed on the shelf 8 cm below the stopping screen shelf and a 10x10 μ m stainless steel screen is used with rupture discs of 650 psi value. After bombardment, tissues are cultured in the dark for one day at 25°C, then transferred to callus maintenance medium 2DG4 + 0.5 mg/l 2,4-D. 10 days later, tissues are transferred onto the same medium supplemented with 100 mg/l PPT. Six to eight weeks later, tissues are transferred onto 40 mg/l PPT. Some of the tissues are then used for inoculation experiments and some of it is transferred on regeneration medium (Murashige and Skoog containing 3% sucrose, 0.25 mg/l ancymitol and 5 mg/l kinetin) with 16 hours of light per day. Transformed plants are identified using the chlorophenol red (CR) assay to test for resistance to PPT (Cramer et al., 1993) and then confirmed by PCR.

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Inoculation of transgenic type I callus with *Agrobacterium*:

Transgenic type I tissues obtained as described above are inoculated with *Agrobacterium*. Maize tissues are soaked in the bacterial suspension for 5-10 min. After inoculation, maize tissues are cultured in the dark at 25°C on 2DG4 + 0.5 mg/l 2,4-D. Two or three days after inoculation, tissues are transferred on the same medium supplemented with cefotaxime (250 mg/l).

Analysis of transgenic material:

Genomic DNA is extracted from 100 mg of callus with the Isoquick^{*} kit (Microprobe, CA) and resuspended in 20 µl water. 1 µl is used for PCR reaction. PCR reactions are performed in Perkin-Elmer^{*} thermal cyclers in a 25 µl reaction using 1x PCR buffer, 0.5 unit of AmpliTaq, 200 µM each dNTPs, 0.2 µM of each primer. To detect the presence of the *pat* gene in tissues, PCR reactions are performed with the PAT primers at an annealing temperature of 55°C. For *iap*, *p35* and *dad-1* gene detection in transgenic tissues, the primers used for each reaction are, P and I, P and 35, P and D at an annealing temperature of 55°C, 55°C, 48°C respectively.

PAT1: 5' - TGTCTCCGGAGAGGAGACC - 3' (SEQ ID NO: 1)

PAT2: 5' - CCAACATCATGCCATCCACC - 3' (SEQ ID NO: 2)

MTL (P): 5' - AGGTGTCCATGGTGCTCAAG - 3' (SEQ ID NO: 3)

iap (I): 5' - ACAATCGAACCGCACACGTC - 3' (SEQ ID NO: 4)

p35 (35): 5' - CCAGGTAGCAGTCGTTGTGT - 3' (SEQ ID NO: 5)

dad-1 (D): 5' - CCTTGTTTCCTTTGTTCACT - 3' (SEQ ID NO: 6)

***p35* and *iap* RT-PCR:**

Total RNA is extracted from transgenic callus using Tripure Isolation Reagent^{*} (Boehringer Mannheim), treated with RNase-free DNase, and 0.5 µg is taken for cDNA synthesis using advantage RT-PCR (Clontech) and oligo-dT primer. After the second-strand synthesis, one twentieth of this reaction is used as a template for PCR reaction with Taq DNA polymerase. *p35* primers used for RT-PCR are:

5' - GGTCCTATACGAAGCGTTAC - 3' (SEQ ID NO: 7) and 5' - CCACGTAGCAGTCGTTGTGT - 3' (SEQ ID NO: 8) amplifying 300 bp of transcript.

iap primers used for RT-PCR are:

5' - CATGTCTGACTTGCGATTGG - 3' (SEQ ID NO: 9) and 5' - ACAATCGAACCGCACACGTC - 3' (SEQ ID NO: 10), amplifying 248 bp of transcript.

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To exclude contamination of genomic DNA, control cDNA reactions in which reverse transcriptase is omitted are prepared in parallel.

Results:

-- Most of embryos and embryogenic calli do not survive the inoculation with *Agrobacterium*. A short exposure (5 min) is sufficient to induce this reaction. Tissues that are cocultivated with *Escherichia coli* and subjected to the same procedures do not exhibit any damage.

-- *p35*, *iap* and *dad-1* were cloned into plant expression cassette between the border sequences of the T-DNA. As these anti-apoptotic genes are carried by the T-DNA, they should be delivered to maize cells at the time of inoculation. Maize tissues are inoculated with *Agrobacterium* LBA4404 (10^9 cells/ml) with or without cell death suppressor genes (between brackets). Following cocultivation, maize embryos or type I callus are examined to quantify tissue survival as an indication of the level of protection conferred by the expression of the anti-apoptotic genes. All treatments are done on about 50 to 80 maize explants and repeated twice. Tissues are transferred 3 days after inoculation on the same medium containing cefotaxime. The effect of *Agrobacterium* is scored two weeks after inoculation. The results are presented in Table 7 and 8, respectively.

Table 7 (embryos)

	No of tissues with response/No of tissues tested (%)	
	Experiment #1	Experiment #2
No bacteria	52/63 (82%)	25/31 (80%)
LBA4404	2/55 (3%)	3/89 (3%)
LBA4404 (<i>p35</i>)	19/75 (25%)	18/74 (24%)
LBA4404 (<i>iap</i>)	22/89 (24%)	21/75 (28%)
LBA4404 (<i>dad-1</i>)	4/48 (8%)	10/66 (15%)

Table 8 (type I callus)

	No of tissues with response/No of tissues tested (%)	
	Experiment # 1	Experiment # 2
No bacteria	76/80 (95%)	72/80 (90%)
LBA4404	8/80 (10%)	6/80 (7.5%)
LBA4404 (p35)	23/80 (29%)	27/80 (33%)
LBA4404 (iap)	25/80 (31%)	30/80 (37%)
LBA4404 (dad1)	15/80 (18%)	13/80 (16%)

About 80% of embryos give rise to embryogenic calli when they are not treated with *Agrobacterium*. About 25% of embryos treated with *Agrobacterium* harboring either *p35* or *iap* give rise to embryogenic callus whereas only 3% of embryos inoculated with *Agrobacterium* exhibited callus initiation. A similar effect is observed with Type I callus inoculated with *Agrobacterium* harboring *p35* or *iap* (Table 2). *dad-1* did only confer low levels of protection in any system.

-- It appears that transient expression of *p35* and *iap* reduce the browning of tissues to some extent but do not completely inhibit the phenomenon. This can be explained by a low efficiency of T-DNA transfer into the maize cells. As judged by transient expression with GUS, it appears that the target tissue is not uniformly transformed. This can be an indication that very few cells are recipients of the T-DNA.

-- The structure of the embryogenic callus emerging from embryos inoculated with *Agrobacterium* containing *p35* or *iap* are not as compact as the control ones. This can be explained by the fact that only cells receiving the T-DNA carrying the anti-apoptotic gene survive and that individual transformed cells do not necessarily transmit the signal to neighbour cells. This type of tissue growth can be evidence that the cell death suppressor genes rescue doomed cells very effectively but their short-term expression does not confer full protection against the effect of *Agrobacterium*.

-- Embryogenic calli transgenic for *p35*, *iap* and *dad-1* result from delivery of said genes under the control of a plant expressible promoter into Type I callus using microprojectile bombardment. The constructs are co-bombarded with the *bar* gene, tissues are selected on PPT and the transformed status of these callus is first confirmed by PCR (Table 9).

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Table 9

Transformation experiment	# PCR +
UbiPAT	5 PAT +
MTLp35, UbiPAT	9 PAT +, 3 p35 +
MTLiap, UbiPAT	7 PAT +, 5 iap +
MTLdad, UbiPAT	11 PAT +, 4 dad-1 +

Sensitivity to *Agrobacterium* inoculation is then assayed on independent events. Callus is inoculated with *Agrobacterium* LBA4404 (10^9 cells/ml). Two independent transgenic callus lines are tested. All treatments are repeated twice. Tissues are transferred 3 days after inoculation on the same medium containing cefotaxime. Survival of maize tissues is scored two weeks after inoculation with *Agrobacterium* (Table 10).

Table 10

Transformation experiment	Number of resistant calli to LBA4404	
	Line1	Line 2
UbiPAT	1/24 (4%)	1/32 (3%)
MTLp35, UbiPAT	22/25 (88%)	25/28 (89%)
MTLiap, UbiPAT	20/22 (90%)	19/25 (76%)
MTLdad, UbiPAT	12/27 (44%)	9/22 (41%)

Embryogenic calli transgenic for *p35* and *iap* do not exhibit cell death when subjected to *Agrobacterium* inoculation whereas a major browning phenomenon is observed on control tissues following cocultivation. The presence of *dad-1* gene delays the onset of apoptosis but does not block cell death as well as *iap* and *p35* do. The results are reproducible in several independent experiments.

-- The protective effect of *p35* and *iap* is also evident when measured by oligonucleosomal DNA fragment electrophoresis.

-- It appears, that the effect of *dad-1* is more obvious when stably expressed in the tissues.

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-- There is a clear correlation between expression of the anti-apoptotic genes and the absence of cell death. *p35* and *iap* genes are expressed in transgenic callus as judged by reverse-transcription-PCR (RT-PCR). Control were uniformly negative.

Example 9: Screening and identification of *Agrobacterium* strains which do not induce cell death in maize cells

The sensitivity of embryogenic callus and embryos of a well studied maize line (A188) and a proprietary elite line (Elite 2) to various *Agrobacterium* strains is tested using a panel of 40 wild-type strains of *Agrobacterium*. These strains are first tested for their compatibility with A188 and Elite 2 embryogenic calli and embryos. Among those, 6 of the 40 strains do not entirely prevent the growth of maize tissues and are further tested on Elite 2 embryos plated on callus initiation medium and inoculated at a concentration of 10^9 cells/ml. Callus initiation is scored 2 weeks after inoculation and expressed as a percentage of embryos with response.

These strains are also tested for their virulence on tobacco leaf discs plated on hormone-free medium, and it is noted that potential of the strains to induce programmed cell death in maize cells does not correlate with the virulence. The percentage in Table 11 describes the number of leaf discs with tumors.

Table 11: Comparison of virulence of non-necrogenic strains

Strains	Source	Opine	Virulence tested on Tobacco (%)	2DG4+5Chl (%)
1	rose	?	100	37
2	peach	nopaline	82	25
3	silver poplar	agropine	66	50
4	chrysanthemum	L,L Sap	100	63
A	rose	nopaline	92	66**
B	salix	nopaline	76	58**

** Good type I callus.

Agrobacterium strains A and B have been deposited with ATCC on May 2, 1997 under the Budapest Treaty under ATCC designation numbers 55964 and 55965 respectively.

Example 10: *Agrobacterium* genes triggering apoptosis in maize cells

A BAC library (bacterial artificial chromosome) is constructed with *Agrobacterium* genomic DNA fragments of approximately 100-200kb in an effort to identify genetic elements responsible for the cell death response in maize embryogenic tissues. The library is introduced into *Escherichia coli*. To check whether such background is suited for screening of the BAC library, *E.coli* containing the BIBAC vector alone is inoculated on maize embryos. *E. coli* is found not to induce a characteristic apoptotic response.

It is estimated that about 25 to 30 BAC clones with a 150-200 kb inserts are sufficient to cover the entire genome of *Agrobacterium*, assuming that *Agrobacterium* has a genome size fairly equivalent to *E. coli* (4.6×10^6 bp). A relatively large number of clones (200) are hybridized with an *Agrobacterium* chromosomal probe (*chvD*) in order to test whether the BAC library made in *E. coli* is a clear representation of the *Agrobacterium* genome. Among 200 clones tested, 5 lighted up with the *chvD* probe (data not shown).

One challenge is that the genetic components from *Agrobacterium* might be silent in *E. coli*. Furthermore, additional factors from plants and/or *Agrobacterium* might be necessary for the expression of such a clone. Therefore, the BAC vector also contains an origin of replication functional in *Agrobacterium* and can be readily transformed into *Agrobacterium* in such a scenario.

Bacterial growth:

Agrobacterium is grown for 3 days on YP medium (5g/l yeast extract, 10g/l peptone, 5g/l NaCl, 15 g/l agar, pH 6.8) supplemented with 100 mg/l kanamycin when needed. Bacteria are collected with a loop and suspended in N6 liquid medium at a density ranging from 10^9 to 5×10^9 cells/ml. *Agrobacterium* cells can also be collected from an overnight culture in YP medium and resuspended in N6 liquid medium.

E.coli DH10B is grown in LB medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 170 mM NaCl, pH 7.0). After transformation, it is grown in SOC solution (2% bacto tryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, pH 7.0).

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Preparation of genomic DNA from *Agrobacterium*:

LBA4404 strain is used for the construction of the library. The protocol, materials and reagents used are from the Imbed^{*} kit (Biolabs, USA). 4 x 10 ml of *Agrobacterium* cultures are grown overnight at 28°C. One hour prior to harvesting, 180 mg/ml of chloramphenicol is added to align the chromosomes. The cells are centrifuged at 800 g at 4°C for 15 min and the pellet is air dried. Cells are pre-warmed in 0.5 ml of cell suspension solution (10 mM Tris-HCl, 20 mM NaCl, 100 mM EDTA, pH 7.2) to 42°C before being embedded in agarose. *Agrobacterium* cells are embedded in agarose rods to carry out the degradation of the cell wall and deproteinisation avoiding shearing of the DNA. To embed the cells in agarose rods, the cells are mixed with an equal volume of 1% low melting point agarose in ddH₂O cooled down to 42°C and then allowed to harden in a Gel Syringe mold. Upon solidification of the agarose, the plugs are transferred to 3 volumes of lysozyme solution (1 mg/ml lysozyme, 10 mM Tris-HCl, 50 mM NaCl, 100 mM EDTA, 0.2% sodium deoxycholate, 0.5% N-laurylsarcosine, pH 7.2) and incubated for 2 hours at 37°C with gentle shaking. The lysozyme solution is removed and the rods are washed twice with 3 ml wash solution for 15 min at a time. The rods are then incubated in 3 ml Proteinase K solution (1 mg/ml proteinase K, 100 mM EDTA, 1% N-laurylsarcosine, 0.2% sodium deoxycholate, pH 8.0) for 20 hours at 42°C with gentle shaking. The Proteinase K solution is removed by aspiration and the rods are dialyzed twice against 5 ml wash solution (20 mM Tris-HCl, 50 mM EDTA, pH 8.0) for 15 min, then with 3 ml PMSF solution (20 mM Tris-HCl, 50 mM EDTA, 1mM phenyl methyl sulfonyl fluoride, pH 8.0) for one hour, followed by two washes with wash solution. The rods are then washed twice with 5 ml storage solution (2 mM Tris-HCl, 5 mM EDTA, pH 8.0) for 15 min and then are reloaded into GelSyringes. Samples 1 mm in length (10-20 ml) are cut with a razor blade and dialyzed against NotI restriction endonuclease buffer (Biolabs, USA) for 30 min at 4°C. The restriction endonuclease buffer is replaced with fresh buffer and incubation is performed overnight at 37°C with 20 units of NotI enzyme (Biolabs, USA). Partial digestion of the *Agrobacterium* DNA is achieved by using NotI, a rare cutting enzyme. NotI theoretically generates fragments 18kb in length after a total digestion assuming the *Agrobacterium* genome has a G/C content of 60% (results extrapolated from different *Agrobacterium* genes). Aliquots are then loaded with a pipette whose tip is cut off. The DNA is separated by pulse field gel (PFG) electrophoresis on a 1% agarose gel in 0.5xTBE at 14°C using a Bio-Rad CHEF Mapper at 6 V/cm for 32 h. The gel is stained for 20 min in 1 mg/ml EtBr ddH₂O bath and agarose slices containing fragments corresponding

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to 100-200kb are excised from the gel. The agarose slices are dialysed twice against TE for one hour at 4°C and twice with agarase buffer (Biolabs, USA) for one hour at 4°C, melted at 65°C for 10 min and digested with one unit of agarase (Biolabs, USA) per 100 mg of agarose. The DNA concentration is estimated fluorometrically using the DyNA Quant 200* (Hoefer, USA).

Preparation of the BIBAC vector:

The BIBAC vector was kindly provided by Dr. Carol M. Hamilton (Cornell University, Ithaca) (Hamilton et al., Proc. Natl. Acad. Sci. (USA) 93, 9975-9979, 1996). BIBAC is designed to replicate both in *E.coli* and *Agrobacterium* and it also contains the *sacBII* gene allowing direct selection for cloned inserts on a sucrose medium. BIBAC vector also harbors the F-factor episome's origin which allows stable maintenance of one or two copies per cell with up to 300kb inserts (Shizuya et al., Proc. Natl. Acad. Sci. (USA) 89, 8794-8797, 1992). The plasmid is isolated using Wizard® Plus Maxiprep kit (Promega, USA). A phenol-chloroform extraction is performed before isopropanol precipitation. The plasmid is digested with NotI and dephosphorylated with 1 unit of shrimp alkaline phosphatase (Boehringer Mannheim) at 37°C for one hour. The ligation is carried out in 40 µl in which 150 ng of *Agrobacterium* DNA is incubated with 17 ng of cut vector (estimated molar ratio 10:1 with vector in excess) with 400 units of T4 DNA ligase (Boehringer) at 17°C overnight. Before transformation, the ligation is dialysed against TE and 1/10 TE in Micro-Collodion* bags (Sartorius, Germany) at 4°C for 2 hours.

Transformation of DH10B with pBAC library

Transformation of competent *E. coli* DH10B cells (F⁻, rec A1; ElectroMax, Gibco-BRL, USA) is carried out by electroporation in pre-cooled Gene Pulser cuvettes (Bio-Rad, USA) with Gene Pulser™ (Bio-Rad, USA) using the following settings: voltage 2.5 kV, capacitance 25 mF, and resistance 200 W. 25 µl of competent cells are mixed with 1 µl of ligation solution for each electroporation. After electroporation, cells are transferred to 0.5 ml SOC solution and incubated at 37°C in a rotating wheel for 45 min. 150 µl aliquots of cells are plated on LB medium containing kanamycin (40 mg/ml) and sucrose (5%).

Isolation of BAC DNA and determination of DNA insert size of BAC clones

BAC clones are grown overnight under continuous agitation in 10 ml LB with kanamycin (50 mg/ml). DNA is extracted according to the alkaline lysis method (Sambrook et al., 1989) with the following modifications: RNase 30 mg/ml is added to the resuspension solution and one phenol-chloroform purification is performed before isopropanol precipitation. The pellet of

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nucleic acid is dissolved in 50 ml of ddH₂O. The DNA is separated by PFG electrophoresis on a 1% agarose gel in 0.5xTBE at 14°C using a Bio-Rad CHEF Mapper at 6 V/cm for 32 h.

Inoculation of maize embryos with *Agrobacterium*:

Immature embryos (0.8 to 2.5 mm) are aseptically excised 12 to 15 days after pollination and plated scutellum up on callus initiation medium (2DG4 + 5 Chloramben) (Duncan's "D" medium with glucose: N6 major, B5 minor, MS iron, 20 g/l sucrose 5 mg/l chloramben, 8 g/l purified agar, G4 additions and 10 mg/l glucose, pH 5.8) (Duncan et al., 1985). Bacteria cells are resuspended into 2N63SM (3.97 g/l N6 salts, N6 vitamins, 30 g/l sucrose, 2 mg/l 2,4-D, 8g/l purified agar, pH 5.8). Embryos are inoculated with *Agrobacterium* or with *E.coli* at a density of 10⁹ cells/ml for 5 min and then cultured on the callus initiation medium for 3 days. Tissues are then transferred on the same medium supplemented with cefotaxime (250 mg/l). Survival of tissues is scored 2 weeks after inoculation.

Transformation of *Agrobacterium*

Agrobacterium transformation is performed as described previously (Wen-Jun and Forde, Nucleic. Acids Research 20: 8385, 1989) in a 0.2 cm electroporation cuvette (Bio-Rad Laboratories Ltd., USA) using a Gene PulserTM (Bio-Rad, USA) at a field strength of 10 kV/cm (2.00 kV), a capacitance of 25 mF and a resistance of 600 Ω.

Vectors for biolistic transformation:

Vectors used to transform maize by the biolistic device are all derivatives of pUC. pMTL is a plant transformation vector containing the maize metallothionein-like gene promoter (MT-L) (de Framond, 1991). The coding region of *virB1* is amplified by polymerase chain reaction with the BAC clone as a template and from strain A with the following primers: 5' - GGAGAGGCGGTGTTAGTT-3' (SEQ ID NO: 11); 5' - CATCATCGCATTCGAGAG-3' (SEQ ID NO: 12). PCR reactions are performed in Perkin-Elmer thermal cyclers, in a 25 µl reaction with 1x PCR buffer, 0.5 unit of AmpliTaq, 200 µM each dNTPs, 0.2 µM of each primer at an annealing temperature of 55°C. The PCR product is first cloned into the pCR2.1 vector for TA cloning (Invitrogen, San Diego, USA). It is then excised by an XbaI-SpeI double digestion and then cloned into the XbaI site of the pMTL vector in sense and antisense orientation giving rise to pMTL-*virB1* and pMTL-*virB1*as respectively. Similarly, the PCR product amplified from strain A is cloned into pMTL in sense and antisense orientation giving rise to pMTL-*virB1*A and pMTL-*virB1*Aas respectively.

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acvB is cloned into the *XbaI*-*XhoI* site of pMTLsense and pMTLantisense, giving rise to pMTL-*acvB* and pMTL-*acvBas*.

pGUS contains the β -glucuronidase gene (GUS) with an intron in the N-terminal codon of the coding sequence and is driven by a chimeric promoter derived from the octopine and mannopine synthase genes (trimer of the octopine synthase promoter upstream activating sequence with a domain of the mannopine synthase promoter) (Ni et al., 1995).

Maize suspension cells

A suspension culture of *Zea Mays* cv Black Mexican Sweet (BMS) is maintained in N6 medium (Chu et al., 1975) and supplemented with 30 g/l sucrose and 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) (2N63S). Maize cell suspensions used for bombardment experiments are taken from 3 day-old rapidly growing cultures. Before bombarding, approximately 0.5 ml of packed volume cells is vacuum filtered onto 7-cm filters (Whatman, N°4). Plated cells are kept 4 hours at 25°C on phytagar-solidified 2N6 medium containing 120g/l sucrose prior to bombardment.

Bombardment of plant cells

Tissues are bombarded with gold microprojectiles onto which a mixture of plasmids is precipitated. pGUS plasmid DNA is used as an internal control in transient expression studies. For co-transformation experiments, the gold particles carry an equal mass of all plasmid DNAs (0.5 μ g of each plasmid DNA per target plate). Appropriate quantities of each DNA are mixed in a total volume of 10 μ l and precipitated with 50 μ l of 2.5 M CaCl_2 and 20 μ l of 0.1 M spermidine-free base onto 50 μ l of 0.3 μ m gold microcarriers (60 mg/ml). Microprojectile bombardment is performed with the PDS-1000 He biolistic device (DuPont) using 1100 psi rupture discs with the sample positioned 8 cm below the stopping screen shelf.

Transient expression assays

β -glucuronidase activity is determined by a chemoluminescent assay with the GUS-Light kit (Tropix). β -glucuronidase activities are expressed as light units detected by an Analytical Luminescence* model 2001 Luminometer integrated over 10 seconds at 25°C.

Colony hybridization

One day old BAC clones are lifted onto nylon filters (Hybond-N, Amersham Life Sciences, UK) for hybridization with radioactive labeled *Agrobacterium* derived probes. DNA probes are labeled with [α - ^{32}P]dCTP using the oligo labeling kit of Pharmacia. The *chvG* probe and

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virD1 probe corresponded to a PCR amplified fragment. The library filters are prehybridized in hybridization solution at 65°C for two hours (5 x SSPE, 5 x Denhardt's solution, 0.5 % SDS 0.1 mg/ml salmon sperm DNA). Hybridization is performed in the same buffer at 65°C overnight. The filters are rinsed in 1 x SSC, 0.1 % SDS for 15 min at 65°C, followed by an incubation in 0.1 x SSC, 0.1 % SDS for 15 min at 65°C. The filters are briefly blotted dry, and film is exposed overnight at -70°C using an intensifying screen.

For PCR amplification, a loop of LBA4404 is resuspended in ddH₂O, heated at 95°C for 15 min, centrifuged and 1 ml is used for PCR reaction with the following conditions: 5 min at 95°C, and then 30 cycles at 95°C for 45 seconds, at 55°C for 45 seconds, and at 72°C for 45 seconds.

Results

- Approximately 20 maize embryos are inoculated with one clone from the BAC library and plated on a callus initiation medium. Following co-cultivation, the embryos are transferred onto the same medium supplemented with 250 mg/ml cefotaxime. Embryos are then examined to assess damage caused by the recombinant bacterium. Among 160 clones screened 4 independent BAC clones are identified (BAC1, BAC2, BAC3, BAC4). To further localize the region on the BAC clone responsible for the cell death of maize tissues, HindIII fragments from BAC clones are subcloned into a bluescript vector. Each of these clones are tested on maize tissues in order to determine which one retains the ability to elicit cell death.
- The DNA sequences are determined by using subclones and oligonucleotides. The sequences of BAC1-2, BAC2-2 and BAC3-2 reveal homology with *virB1*, *xylA-xylB* and *acvB* respectively. BAC-4 is identical to BAC3-2.
- BAC clones harboring *acvB* (BAC3 and BAC4), *virB1* (BAC1) and *xylA-xylB* (BAC2) are introduced into strain A resulting in strains A(*acvB*), A(*virB1*) and A(*xylA*). These strains are used to inoculate maize tissues. Tissues inoculated with LBA4404 display a typical cell death pattern whereas the infection with strain A fails to produce these symptoms. A(*acvB*), A(*virB1*) and A(*xylA*) induce cell death at different levels, with A(*acvB*) being the strongest one. The presence of these genes thus converts the interaction of strain A with maize and renders the maize tissues more sensitive to infection (Table12).

Table 12

Strains	Exp 1	Exp2	Exp3
No Bacteria	19/22 (86%)	15/18 (83%)	16/18 (88%)
LBA4404	2/20 (10%)	1/17 (6%)	3/20 (15%)
E.coli	17/20 (85%)	16/19 (84%)	17/21 (81%)
Strain A	19/20 (95%)	17/21 (81%)	17/20 (85%)
E.coli (BAC1)	5/20 (25%)	5/17 (30%)	4/18 (22%)
E.coli (BAC2)	10/19 (52%)	10/20 (50%)	9/19 (47%)
E.coli (BAC3)	3/18 (16%)	4/17 (24%)	3/20 (15%)
E.coli (BAC4)	4/19 (21%)	3/19 (16%)	2/21 (9%)
Strain A (BAC1)	5/19 (26%)	6/17 (35%)	8/25 (32%)
Strain A (BAC2)	9/22 (41%)	10/23 (43%)	8/19 (42%)
Strain A (BAC3)	3/20 (15%)	2/18 (11%)	3/21 (14%)
Strain A (BAC4)	2/20 (10%)	3/19 (16%)	4/20 (20%)

- In Southern analysis, HindIII-digested genomic DNA from *Agrobacterium* strains A136, LBA4404, LBA4402, A, and B is probed under conditions of moderate stringency with either the fragment carrying *xyIA* or *acvB*. Homologous fragments to *xyIA* are present in all strains tested. Sequences hybridizing with *acvB* are found in LBA4404, LBA4402 and A136 that induce cell death in maize cells. Of interest, *acvB* is lacking in *Agrobacterium* strains A and B. *acvB* is thus limited in its distribution. As previously reported, *acvB* is present in relatively few strains of *Agrobacterium* (Wirawan et al., Biosci. Biotech. Biochem. 60: 44-49, 1993). *xyIA* and *acvB* are not carried by the Ti plasmid because they were present on the Ti-cured strain LBA4402.
- To determine whether expression of *acvB* and *virB1* within maize cells is lethal, these genes are cloned into a plant expression vector and co-transformed with the pGUS vector expressing β -glucuronidase (GUS). In case the test gene is lethal, GUS activity is reduced or eliminated (Mindrinos et al., 1994). The coding region of the two genes is subcloned in sense and antisense orientation into a plant expression vector containing the MTL promoter and biolistically delivered into maize cells along with the vector pGUS. After 30 hr the activity of GUS is assessed in maize cells. Results are shown in

Table 13. The GUS activity is reduced about two fold when virB1 is co-bombarded with pGUS. Maize cells co-bombarded with *acvB* and pGUS consistently show a 35-fold reduction in GUS activity. This indicates that the expression of *acvB* at high levels within maize cells is deleterious.

Table 13

The plasmid constructs are delivered to maize cells by the biolistic device. Following incubation for 30 hr, tissues are homogenized and GUS activities are determined. Activities are expressed as Light Units/ μ g protein. Independent bombardments are analyzed, and data are presented as mean values of five repetitions \pm SD.

Plasmids + pGUS	Mean \pm SD (Light Units/ μ g protein)
pUC	2082 \pm 199
pMTL-virB1	1014 \pm 106
pMTL-virB1as	2543 \pm 176
pMTL-virB1K6	1738 \pm 330
pMTL-virB1K6as	2168 \pm 282
pMTL-acvB	61 \pm 19
pMTL-acvBas	2378 \pm 292

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: NOVARTIS AG
- (ii) TITLE OF INVENTION: PLANT TRANSFORMATION METHODS
- (iii) NUMBER OF SEQUENCES: 12
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: FETHERSTONHAUGH & CO.
 - (B) STREET: P.O. BOX 2999, STATION D
 - (C) CITY: OTTAWA
 - (D) STATE: ONT
 - (E) COUNTRY: CANADA
 - (F) ZIP: K1P 5Y6
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: ASCII (text)
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: CA
 - (B) FILING DATE: 29-MAY-1998
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/867,869
 - (B) FILING DATE: 02-JUN-1997
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: FETHERSTONHAUGH & CO.
 - (B) REGISTRATION NUMBER:
 - (C) REFERENCE/DOCKET NUMBER: 21489-9559
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (613)-235-4373
 - (B) TELEFAX: (613)-232-8440

38a

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: PAT1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TGTCTCCGGA GAGGAGACC

19

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

WO 98/54961

PCT/EP98/03215

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(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: PAT2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CCAACATCAT GCCATCCACC

20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: MTL (P)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AGGTGTCCAT GGTGCTCAAG

20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

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(B) CLONE: iap (I)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ACAATCGAAC CGCACACGTC

20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: p35 (35)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CCAGGTAGCA GTCGTGTGTGT

20

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: dad-1 (D)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCTTGTTCCTC TTGTTCCTC

20

(2) INFORMATION FOR SEQ ID NO: 7:

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- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (iii) HYPOTHETICAL: NO

- (iii) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GGTCCTATAC GAAGCGTTAC

20

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (iii) HYPOTHETICAL: NO

- (iii) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CCACGTAGCA GTCGTTGTGT

20

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (iii) HYPOTHETICAL: NO

- (iii) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

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CATGTCGTGAC TTGCGATTGG

20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ACAATCGAAC CGCACACGTC

20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GGAGAGGCCGG TGTTAGTT

18

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

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(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CATCATCGCA TTCGAGAG

18

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CLAIMS:

1. A method of making a transgenic plant cell,
comprising:

(a) transforming a plant cell with a gene of interest by co-
5 cultivating said plant cell with *Agrobacterium* under
conditions which inhibit *Agrobacterium* Induced Necrosis
(AIN), wherein said *Agrobacterium* comprises a vector
comprising said gene of interest and wherein said conditions
are selected from the group consisting of:

10 (i) co-cultivating said plant cell with
Agrobacterium after heat-shock treatment of said plant cell;
and

(ii) co-cultivating said plant cell with
Agrobacterium, wherein said plant cell has been transformed
15 with a nucleotide sequence which encodes an mRNA or protein
from a gene selected from the group consisting of:
baculovirus p35, baculovirus inhibitors of apoptosis (iap),
and dad-1, in operative linkage with a promoter; and

to produce a transgenic plant cell having a
20 transgene comprising said gene of interest;

(b) selecting said transgenic plant cell.

2. A method according to claim 1 wherein said heat-
shock treatment is performed at 40-50°C.

3. A method according to claim 2 wherein said
25 treatment is performed at 42-48°C.

4. A method according to claim 2 or claim 3 wherein
said treatment lasts for 2 to 10 minutes.

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5. A method according to any one of claims 1 to 4 wherein said plant cell is a *Gramineae* sp. cell.

6. A method according to claim 5 wherein said cell is a maize or wheat cell.

5 7. A transgenic plant cell produced according to the method of claim 1, wherein the plant cell comprises a nucleotide sequence which encodes an mRNA or protein from a gene selected from the group consisting of: baculovirus p35; baculovirus inhibitor of apoptosis (iap); and dad-1.

10 8. A transgenic plant cell according to claim 7 which is a maize or wheat cell.

9. A method of making a fertile transgenic plant comprising:

(a) transforming plant tissue by co-cultivating said plant
15 tissue with *Agrobacterium* under conditions which inhibit *Agrobacterium* Induced Necrosis (AIN), wherein said *Agrobacterium* comprises a vector comprising a gene of interest and said conditions are selected from the group consisting of:

20 (i) co-cultivating said plant tissue with *Agrobacterium* after heat-shock treatment of said plant tissue; and

(ii) co-cultivating said plant tissue with *Agrobacterium*, wherein cells of said plant tissue have been
25 transformed with a nucleotide sequence which encodes an mRNA or protein from a gene selected from the group consisting of: baculovirus p35, baculovirus inhibitors of apoptosis (iap), and dad-1, in operative linkage with a promoter;

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to produce a transgenic plant tissue, the cells of which have a transgene comprising said gene of interest; and

(b) regenerating said transgenic plant tissue to produce said fertile transgenic plant.

5 10. A method according to claim 9 wherein said heat-shock treatment is performed at 40-50°C.

11. A method according to claim 10 wherein said treatment is performed at 42-48°C.

12. A method according to claim 10 or claim 11 wherein
10 said treatment lasts for 2 to 10 minutes.

13. A method according to any one of claims 9 to 12 wherein said plant tissue is from *Gramineae sp.*

14. A method according to claim 13 wherein said plant tissue is from maize or wheat.

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OTTAWA, CANADA

PATENT AGENTS