



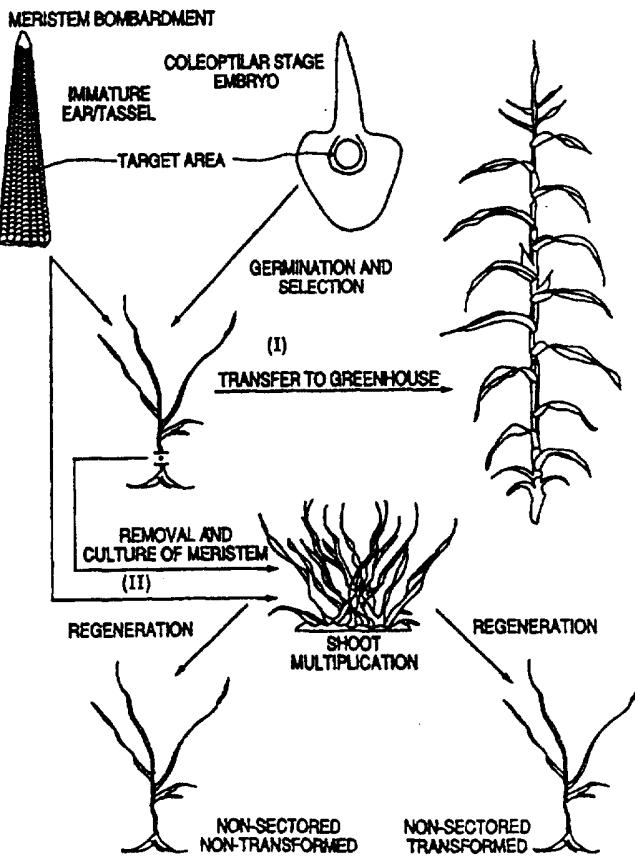
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## (54) Title: TRANSGENIC CEREAL PLANTS

## (57) Abstract

To obtain a transgenic cereal plant which is stably transformed, an exposed cereal meristem is subjected to biolistic bombardment in order to target non-differentiated meristem cells for transformation. Immature embryos at the early proembryo, mid proembryo, late proembryo, transitional or early coleoptilar stage are harvested for biolistic bombardment. The meristem tissue or cells fated to contribute to the meristem then are manipulated in order to enlarge transgenic sectors, either through selection and/or through effecting a proliferation from the tissue of shoots or multiple meristems *per se*. The shoot population thus obtained then is screened, by means of a nonlethal enrichment assay, to identify either chimeric sectors that will contribute to germline transmission, or non-sectored, L2 periclinal chimeras that will by definition transmit to progeny. Increased time in culture, under selection, enhances the prospects for sectoral-to-periclinal conversions, and also selects for L1-to-L2 conversions which, through a shift in position, ultimately contribute to the germline. Transgenic sectors also are stabilized during the step of tillering.



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## TRANSGENIC CEREAL PLANTS

BACKGROUND OF THE INVENTION

The present invention relates to obtaining plants by a methodology that entails the biolistic bombardment of 5 meristem tissue, at a very early stage of development, and the selective enhancement of transgenic sectors, toward genetic homogeneity, in cell layers that contribute to germline transmission.

Production of transgenic plants first became routine 10 through the use of *Agrobacterium*, and the use of this vector with totipotent tissues has become the method of choice for many dicotyledonous species. While steady progress has been made in expanding the genotype and species range of this method, *Agrobacterium*-mediated 15 transformation has not been widely utilized for monocotyledonous species, including cereals, and is likely in the near term to remain restricted to specific genotypes. Similarly, protoplast-based methods are not widely applicable for monocots.

20 The first reports which appeared on biolistics-mediated production of fertile, transgenic maize were restricted to a specific hybrid, A188 x B73. See Gordon-Kamm et al., *Plant Cell* 2: 603 (1990), and Fromm et al., *Bio/Technology* 8: 833 (1990). Since then the technique 25 has been extended to many important monocot crops, including barley, wheat, rice and oats, and the useful range in maize has been expanded slowly to include a handful of genotypes, for example, the commonly used A188xB73, H99, FR16 and Pa91 genotypes. This work 30 generally has revolved around a common theme, which is the initiation of regenerable callus from the scutellum of the embryo. In particular, all the reports in this context have highlighted a prerequisite of initiating regenerable callus from the scutellum of the immature 35 embryo, regardless of whether there is bombardment (i) of the scutellum just after embryo isolation, followed by selection of the callus grown from the scutellar cells, (ii) of freshly initiated callus after a short pre-

culture of the scutellum or (iii) of long-term callus or cell suspension cultures.

Progress in expanding the callus-based approaches to new genotypes or species has occurred via adaptations of 5 the basic method to accommodate differences in morphology and growth patterns that typify different forms of immature, embryo-derived callus, i.e., friable callus versus compact callus, also referred to as Type II and Type I, respectively. Genotype restrictions remain, 10 however, because some germplasm does not produce an appropriate callus response.

With the advent of biotics-mediated transformation, numerous groups have explored the possibility of using microprojectile-delivery methods 15 with meristem tissues. It has remained "an open question," however, as to "whether integrative transformation in cells of the shoot apical meristem of [a] monocotyledonous species is [even] possible." Bilang et al., *Plant J.* 4: 735 (1993).

20 The literature is marked by speculation concerning barriers to transforming meristem target cells which may explain the lack of success in this area. It has been observed, for example, that cereal "shoot meristems are tiny (about 100  $\mu\text{m}$ ) and...biolistic particles hit large 25 target areas at random," and that "meristematic cells may [have] molecular mechanisms which prevents [sic] integration of foreign DNA...." Potrykus, *Nature* 355: 568, 569 (1992). More generally, the fact that monocot plant species tend to display less developmental 30 plasticity than dicot species has engendered an expectation that monocots are less amenable to stable transformation by biolistic and other techniques.

Given the lack of developmental plasticity in cereals, therefore, the historical focus of 35 transformation efforts in these crops has been on callus derived from one of the few genotypes that produce Type I or Type II embryogenic callus. These transformation targets were subject to easy use because a large

population of undetermined, proembryogenic cells could be selected. Accordingly, many research groups have taken advantage of this approach and have not pursued alternative target tissues. In particular, no one to 5 date has reported germline transformation via meristem bombardment of maize, a key cereal crop. Lack of success in this regard has been ascribed to the rigid developmental fate of the cells composing the meristem.

SUMMARY OF THE INVENTION

10 It is therefore an object of the present invention to provide a methodology for the reproducible production of stably transformed cereal plants.

15 In accomplishing this object and others, there has been provided, in accordance with one aspect of the present invention, a method for producing transgenic cereal plants, e.g., maize, sorghum, wheat, barley, oat or rice plants, that will transmit introduced DNA to progeny, comprising the steps of

20 (A) introducing foreign DNA into cells selected from the group consisting of (i) cells of a meristem that is not enclosed by sheathing leaves and (ii) cells fated to contribute to said meristem; then

25 (B) inducing reorganization of said meristem to increase transgenic sector size, whereby the likelihood that a transgenic sector will contribute to germline transmission is increased; and thereafter

30 (C) exposing said meristem to conditions under which it differentiates to form a plantlet, wherein said plantlet contains said transgenic sector or is homogeneously transformed by said foreign DNA, such that said plantlet can be grown into a transformed cereal plant that will transmit said foreign DNA to progeny.

35 The foreign DNA can be introduced into a plurality of meristems, at least some of which differentiate in step (C) to form a plurality of plantlets. The foreign DNA is introduced into a meristem that is not enclosed by sheathing leaves including meristems from early

proembryo, mid proembryo, late proembryo, transitional and early coleoptilar stage embryos.

In one preferred embodiment, reorganization is effected through at least one manipulation selected from

5 the group consisting of (i) imposition of a nonlethal selective pressure on the meristems, (ii) mechanically-induced meristem reorganization, and (iii) hormonally-induced shoot multiplication. In another preferred embodiment the conditions in step (C) are such that the

10 meristems undergo maturation and plant differentiation to form shoot apices, and the method further comprises effecting reorganization of meristem tissue in the shoot apices to enlarge transformed sectors or to produce periclinal L2 chimeras. The reorganization in this

15 regard can be effected, for example, by exposing the shoot apices to nonlethal selection pressure such that transformed cells have a competitive growth advantage over nontransformed cells in the shoot apices, and the proportion of transformed cells in the shoot apices is

20 increased. In yet another preferred embodiment, the method further comprises a step before step (B), e.g., before step (A), of wounding the apical dome selectively. A method of the present invention also can comprise the

25 further steps of (i) dissecting out an axillary bud from above the base of a leaf of a plantlet when a chimeric sector is observed in a substantial portion of the leaf, and then (ii) germinating the axillary bud into a whole plant or subjecting the axillary bud to shoot multiplication.

30 In yet another preferred embodiment, the transgenic sector of a plantlet is stabilized by inducing tillers. The apex of a transgenic plantlet is removed, the wounded plantlet is grown to induce formation of a plurality of tillers, and transgenic tillers then are selected from

35 that plurality.

In accordance with another aspect of the present invention, a transgenic cereal plant is provided that (A) is the product of a method as described above,

(B) transmits introduced DNA to progeny and (C) belongs to a cereal line that is recalcitrant to callus-based transformation. In a preferred embodiment, the transgenic cereal is a maize plant that is not produced by transformation of a genotype selected from the group consisting of A188, A188 x B73, H99, Pa91, FR16 and a genotype obtained from a cross involving any of the foregoing.

According to still another aspect of the present invention, a maize plant is provided that transmits introduced DNA to progeny and that has a pedigree including a line selected from the group consisting of PHT47, PHP02, PHV78, PHK05, PHW20, PHR62, PHN37, PHM10, PHV37, PHJ65, PHBW8, PHK29, PHJ33, PHP60, PHN73 and PHHV4.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only. Indeed, various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

#### 25 BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a series of line drawings depicting the structure of a typical cereal embryo (a) at the coleoptilar stage, which in maize occurs approximately 8 to 14 days after pollination, and (b) at the later third stage (about 22 to 28 days after pollination in maize), respectively; (c) the model shoot tip of an angiosperm, including cereals, shown in longitudinal section; and (d) the shoot and root structures which pertain in cereals generally, with a unit phytomer of the shoot highlighted. Abbreviations: c = coleoptile; cn = coleoptilar node; cp = coleoptilar pore; cr = coleorrhiza; m = mesocotyl; r = primary root primordium; s = suspensor; sa = shoot apex;

sc = scutellum; scn = scutellar node; sr = seminal root primordium.

FIGURE 2 is a schematic representation of transformation methodology according to the present  
5 invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

It has been discovered that the difficulties discerned previously in relation to applying a meristem-based transformation strategy to cereals can be overcome  
10 by (A) biolistically targeting cells of the shoot apical meristem under conditions such that the apical dome of the apical meristem is not enclosed by sheathing leaves, as depicted in FIGURE 1; and (B) using a nonlethal selection regimen to effect an enrichment of transformed  
15 cells, for example, by giving transformed cells a competitive advantage over nontransformed cells, and thereby to facilitate an increase in sector width. The nonlethal selection also can promote the development of a short-lived, mericinal L2 event into a stable, periclinal event in which most or all of the cells contributing to the germ line (i.e., the L2 layer) are transformed.  
20 In addition, it has been found that selective pressure can promote L1- to-L2 conversion events, thus increasing the probability of germ line transmission.  
25

The shoot apical meristem in cereals is highly variable between species. In most species, however, a stratified meristem exists that is composed of two or three visible layers which generate the entire shoot: a superficial L1, a subsurface L2 and, in some cases, a deeper L3 layer. The outer layer(s) comprise the tunica, which are characterized by anticlinal cell divisions. In contrast, divisions in the innermost layer, the corpus, occur randomly, both anticlinally and periclinally. In maize the meristem is believed to be composed of only two layers, L1 and L2, and possibly a third L3 layer. See Poethig, CONTEMPORARY PROBLEMS IN PLANT ANATOMY 235-39  
30 (1984). Cell differentiation to delimit the major  
35

tissues of the shoot is position-dependent rather than lineage-dependent.

For example, in most species the epidermis is generated almost exclusively by the L1 layer, with the L2 layer contributing to the germ line. In the process of introducing a foreign gene into a subset of apical meristem cells, one creates a plant that necessarily is "chimeric," i.e., a plant in which portions have been altered in genetic composition. There are three major categories of chimeric plants, based on the characteristic pattern of genetic differences:

(1) **sectoral chimeras**, in which a portion of the plant is "genetically distinct" through all cell layers by virtue, for example, of displaying a mutant somatic phenotype, a change in chromosome number, or the presence of transformed cells; (2) **periclinal chimeras**, in which an entire cell layer (L1 alone or L2 alone, for instance) is different from the rest of the plant; and (3) **mericlinal chimeras**, which represent an intermediate between the other two types, i.e., a genetic difference characterizes only a portion of one layer.

In this description, the terms "biolistic" and "biolistically" denote an approach to genetic transformation described, for example, in U.S. patents No. 4,945,050 and No. 5,141,131, the respective contents of which are hereby incorporated by reference.

Pursuant to a ballistic approach, force is transmitted to small particles that carry DNA, for example, coated on particulate surfaces or absorbed into the particles, in such a way that the exerted pressure forces particles into a targeted cell or tissue ("biological sample"); the particles thus are called "microprojectiles" or "microcarriers." In other words, the microprojectiles are propelled at the biological sample, accelerating to such speed that, upon impact, they penetrate cellular surfaces and are incorporated into the interior of a cell or cells in the sample.

The microprojectiles should have an average diameter sufficiently small to permit penetration of and retention by cells of the biological sample without killing the cells. Particles of gold or tungsten, in the size range 5 of about 0.1 to 4 microns, are illustrative of microprojectiles that are suitable for delivering exogenous nucleic acid into a host. Other types of biolistic delivery vehicles are disclosed, for example, by U.S. patents No. 5,120,657 (electrical discharge 10 propels a carrier sheet toward target) and No. 5,240,842 (nucleic acid delivered via aerosol droplets), and in PCT application WO 92/01802 (ice particles as carrier).

In relation to aspect (A) mentioned above, the present invention contemplates the biolistic targeting of 15 apical meristem cells at an early developmental stage. In a preferred embodiment, meristem cells are bombarded at a developmental phase that is no later than the coleoptile-ring stage, when the apical dome is fully exposed, lacking protection from leaf primordia, and is 20 composed of fewer cells in the meristem than are present at later stages. The stages of maize embryogenesis are described in detail by Poethig et al., *Developmental Biology* 117: 392-404 (1986), the contents of which are incorporated by reference.

25 More specifically, the transformation method of the present invention focuses on coleoptilar and earlier stage embryos, namely, early proembryo, mid proembryo, late proembryo, and the transitional stages of embryo development. At the earliest stages of development, the 30 meristem is not defined; instead, a group of cytoplasmically dense cells undergo more rapid division and, ultimately, form the apical meristem.

In targeting these various embryo stages, therefore, 35 DNA is introduced (i) into cells that make up the meristem proper (i.e., at the coleoptilar stage) or, (ii) in the earlier stages of development, into cells that are destined, by position or fate, to contribute to the meristem. Biolistic bombardment according to the present

invention is effected by orienting the embryo so that cells that are within a meristem or that are destined to contribute to the meristem are exposed directly to the biolistic projectiles.

5        In late proembryos, the axis side of the embryo is slightly flattened, allowing this side of the embryo to be placed face up (away from the agar) for bombardment. Transition stage and coleoptilar stage embryos are similarly oriented. There is no such orientation, 10 however, for mid and early proembryos on agar after isolation (i.e., before shooting). Rather, when proembryos are placed in a random orientation on the agar medium, the meristem apparently develops on the upper side of the embryo (away from the medium). Thus, 15 placement on the medium may be stimulating the embryo to re-orient its growth axis, for example, by virtue of the *in vitro* conditions which are provided (i.e., the new hormonal gradient that is being established within the embryo).

20        A convenient and, hence, preferred source of meristems for use in the present invention are coleoptilar stage embryos. At the coleoptilar stage of cereal embryo development, the coleoptile is visible as a ring of leaf primordium surrounding an exposed 25 meristem. In maize, the early coleoptilar stage can generally be obtained 10 to 12 days after pollination. (The days-after-pollination criterion, or "DAP," is affected by embryonic environment and genotype, and therefore is an adjunct to developmental staging based on 30 morphology, which is an important criterion for timing of transformation in the present invention.) At the early coleoptilar stage the boundary of the meristem is distinct, with a visible tunica and corpus (L1 and L2 layers, respectively).

35        A particularly preferred source of target cells for use in the present invention are present in the early proembryo, mid proembryo, late proembryo and the transitional stages in embryo development. In maize, the

early proembryo, mid proembryo, late proembryo and the transitional stages can generally be isolated 2, 4, 7-8 and 8-10 DAP, respectively (see Poethig et al. (1986), cited above). Again, the developmental stage is the 5 important criterion. Rate of development and, hence, DAPs at which embryos are isolated vary with growth environment and genotype.

At the mid proembryo stage there is no distinction 10 between the L1 and L2 layers. The distinction between L1 and L2 progresses until it is well-defined by the time the embryo reaches the transitional stage.

Alternatively, immature staminate inflorescences 15 (tassels) and pistillate inflorescences (ears) can serve as sources of meristems for transformation in accordance with the present invention. "Immature" here denotes a developmental state when floral meristems still are developmentally plastic, i.e., are capable of shoot differentiation. This developmental plasticity should be exploitable, pursuant to the present invention, for 20 transformation of many Graminaceous species, given the recognized similarities in inflorescence development among the grasses.

A trained technician can isolate 200 to 600 mid 25 proembryo, late proembryo, transitional or coleoptilar-stage embryos per day, the ease of isolation and number of isolated embryos increasing with embryo size. On the order of ten times as many meristem explants can be isolated from immature tassels and/or ears, and a large 30 percentage of these can be induced to follow a vegetative pattern of development. Another important advantage associated with using floral explants as meristem sources is that many genotypes exhibit better meristem growth and 35 shoot multiplication when the floral explants are the starting material. This advantage is pronounced, for example, with respect to an inbred maize line with a lineage that includes line PHV78. Conversely, immature embryos are the preferred explant for some genotypes, such as maize inbreds having a lineage including line

PHBW8. Access to both options significantly extends the genotype range for meristem transformation pursuant to the invention.

Whatever explant or tissue is used as a source of target cells for biolistic treatment in accordance with the present invention, the bombarded cells are subjected to a first, nonlethal selection pressure in the course of generating plantlets which are grown out directly (course I in FIGURE 2) or, alternatively, are subjected to meristem reorganization, induced mechanically or hormonally, in advance of a second nonlethal selection (course II in FIGURE 2). The aspects of nonlethal selection and induced meristem reorganization are addressed in greater detail below.

The developmental fate of cells within the meristem normally is rigidly determined. Thus, transformation of a particular cell within the meristem typically will result in a small transgenic sector made up only of the descendants of that cell. Without further manipulation, such sectors rarely if ever overlap gametophytic tissue during normal development. But by targeting cells at earlier developmental stages, as described above, and then applying mild selective conditions in accordance with the present invention, i.e., the pressure provides a growth advantage to transformed cells but is not severe enough to impede the overall development of the meristem, then the consequent faster division rate of transformed cells results in the descendent cells comprising a greater portion of the meristem. Accordingly, the transgenic sector contributes to a larger portion of the mature plant, and there is a greater likelihood that the sector will contribute to germline transmission.

According to one preferred embodiment, a selective growth advantage is imparted to transformed cells in the form of NPTII-encoded resistance to tobramycin, kanamycin or a related compound. It is acceptable, however, to confer resistance to another "bleaching" antibiotic (by means of a streptomycin-resistance gene, for instance) or

herbicide, for example, by transformation with the *crtI* gene, which imparts resistance to norflurazon. By the same token, the present invention contemplates similar non-lethal strategies which entail the use of other 5 selective agents, such as bialaphos and hygromycin, with a corresponding, resistance-imparting gene, so long as the resulting selective pressure retards the growth of non-transformed cells relative to cells in the transgenic sector.

10 A model experiment in this regard would involve exposing samples of isolated meristem tissue to a graded series of dilutions of the selection agent in the medium of choice, and then determining a concentration threshold below which the selective pressure favoring transformed 15 cells is not so stringent as to be detrimental to general meristem development. While this approach typically would result in continued meristem growth during selection, the present invention also envisages establishing conditions of little or no meristem growth 20 ("static conditions") which are punctuated by brief exposure(s) to a higher concentration of selection agent ("pulsed selection") which otherwise would adversely affect overall meristem development.

As noted above, cereal transformation according to 25 the present invention optionally involves a reorganization of the meristem, for example, by wounding of the apical dome. While other methods of wounding also result in reorganization, a preferred method is to pierce the apical dome using a micromanipulation needle. The 30 reorganization thus effected alters growth in the apical dome and, it has been discovered, prompts a proliferation of multiple meristems which, in turn, enhances transformation frequency and sector size. For example, mechanically-induced meristem proliferation in 35 conjunction with selective pressure results in an increase in frequency and size of the transgenic sectors observed in subsequent leaves.

Meristem reorganization may precede biolistic treatment, followed by germination and selection leading to the production of chimerically transformed plants (course I). Alternatively, mechanical wounding can be 5 performed after bombardment of the meristems in order to effect a proliferation of meristems. When applied in this manner on chimeric meristems, the sectors can enlarge because the reorganized meristems are derived from a smaller number of cells and, hence, the percentage 10 of transformed cells in the meristems is increased.

Pursuant to course II (see FIGURE 2), a reorganization is brought about by hormonally-induced shoot multiplication with respect to the developing shoot meristem of a plantlet selected for the presence of a 15 transformed sector. The hormonally induced reorganization need not be exclusive of the optional, mechanically induced reorganization mentioned above, and brings about meristem proliferation via shoot multiplication.

20 To effect hormonally induced reorganization, the developing shoot meristem first is localized, typically in a swelling that occurs in the germinated plantlet at the junction between the mesocotyl and the epicotyl (see FIGURE 2). A section of 2 to 3 mm in size which contains 25 the meristem then can be excised at the swelling point and cultured on a shoot proliferation medium of the sort described, for example, by Lowe et al., *Plant Science* 41: 125 (1985), and by Zhong et al., *Planta* 187: 483 (1992), respectively. To this end, meristems typically are 30 cultured on MS medium with 2 mg/l BAP (6-benzyl-aminopurine), 3% sucrose and 9 mg/l agar. More generally, a shoot multiplication medium will utilize a cytokinin, such as Kinetin, BAP, Thidiazuron or Zeatin, at a concentration between 0.5 and 10 mg/l. A low level 35 of auxin also may be required in some genotypes. Murashige and Skooge (MS) salts are adequate but probably not optimal, in that preliminary experiments using media with ammonium levels higher than those in MS resulted in

an improved culture response. Additional additives such as the auxin transport inhibitor, TIBA, and ethylene inhibitors like silver nitrate and cefotaxime also appear to be beneficial.

5 By virtue of its hormonal constituency, the shoot proliferation medium forces the generation of a few to hundreds of shoots from each excised shoot meristem, thereby increasing the likelihood of obtaining a subpopulation of shoots, some of which may arise from a  
10 transformed sector. Unlike mericinal and sectoral chimeras, which exhibit a lower probability of germline transmission, a significant and reproducible percentage of the resulting shoots are periclinal chimeras and, hence, are "stabilized" in the sense that genetic  
15 homogeneity is promoted within a cell layer, such as the L2 layer, that ultimately contributes to germline transmission.

20 To identify the aforementioned shoot subpopulation, the large population of induced shoots is screened to identify non-sectored, periclinal chimeras. This is accomplished via a nonlethal assay which brings about an enrichment of transformed cells through the use of  
25 selective agents (i) that bleach normally green tissue at levels that do not inhibit growth or (ii) that inhibit growth of non-transformed meristem sectors without significantly reducing viability of the meristems.

30 Use of an appropriate selective agent at nonlethal levels, as described, also provides the opportunity to assess visually the extent of homogeneity within a transformed meristem layer. Increased time in culture under selection, pursuant to the present invention, enhances the prospect of mericinal-to-periclinal conversions and of sectoral-to-homogeneously transformed conversions, and also selects for L1-to-L2 conversions  
35 which, through a shift in position, ultimately contribute to the germline.

From the preceding commentary it is apparent that one aspect of the present invention relates to forcing

meristem reorganization, before bombardment, after bombardment or both, by suppressing cell growth through selective wounding of the apical dome, prompting generation of multiple meristems, or by exposing excised 5 meristems to hormonal stimuli likewise leading to multiple meristems, albeit in the form of proliferated shoots. According to yet another preferred embodiment, the axillary bud of a transformed plantlet can be dissected out, from just above a leaf base, when a 10 chimeric sector is observed in a substantial portion of that leaf. The isolated axillary bud represents an additional meristem that can be grown into a whole plant, or taken through a brief cycle of shoot multiplication as described, thereby to obtain a more homogeneously 15 transformed plant.

The purpose of this approach, as for the others discussed above, is to increase the frequency of germline transmission. Thus, if a transformed sector runs through more than one leaf, it should be possible to "capture" 20 that transformation event in a axillary bud, i.e., convert a transformed mericinal or sectoral chimera into a periclinally or homogeneously transformed shoot.

Another method of stabilizing transgenic sectors is to induce tillers in the transformed plant. In those 25 cases in which transgenic sectors are limited to the lowermost leaves or domains of maize plants, tillering is induced to stabilize these transgenic sectors.

By means of the present invention, a wide range of cereal varieties can be transformed stably, in a 30 genotype-independent manner, for the first time. In maize, for example, this means that elite lines which were previously inaccessible to transformation characterized by transmission of imparted trait(s) to seed progeny now can be genetically engineered to express 35 various phenotypes of agronomic interest. The genes implicated in this regard include but are not limited to those categorized below.

I. Genes That Confer Resistance To Pests or Disease And That Encode:

5 (A) A *Bacillus thuringiensis* protein, a derivative thereof or a synthetic polypeptide modeled thereon. See, for example, Geiser et al., Gene 48: 109 (1986), who disclose the cloning and nucleotide sequence of a *Bt*  $\delta$ -endotoxin gene. Moreover, DNA molecules encoding  $\delta$ -endotoxin genes can be purchased from American Type 10 Culture Collection (Rockville, MD), under ATCC accession Nos. 40098, 67136, 31995 and 31998.

15 (B) A lectin. See, for example, the disclosure by Van Damme et al., *Plant Molec. Biol.* 24: 825 (1994), who disclose the nucleotide sequences of several *Clivia miniata* mannose-binding lectin genes.

20 (C) A vitamin-binding protein such as avidin. See U.S. patent application serial No. 07/911,864, the contents of which are hereby incorporated by reference. The application teaches the use of avidin and avidin homologues as larvicides against insect pests.

25 (D) An enzyme inhibitor, for example, a protease inhibitor or an amylase inhibitor. See, for example, Abe et al., *J. Biol. Chem.* 262: 16793 (1987) (nucleotide sequence of rice cysteine proteinase inhibitor), Huub et al., *Plant Molec. Biol.* 21: 985 (1993) (nucleotide sequence of cDNA encoding tobacco proteinase 30 inhibitor I), and Sumitani et al., *Biosci. Biotech. Biochem.* 57: 1243 (1993) (nucleotide sequence of *Streptomyces nitrosporeus*  $\alpha$ -amylase inhibitor).

35 (E) An insect-specific hormone or pheromone such as an ecdysteroid and juvenile hormone, a variant thereof, a mimetic based thereon, or an antagonist or agonist thereof. See, for example, the disclosure by Hammock et al.,

Nature 344: 458 (1990), of baculovirus expression of cloned juvenile hormone esterase, an inactivator of juvenile hormone.

5 (F) An insect-specific peptide or neuropeptide which, upon expression, disrupts the physiology of the affected pest. For example, see the disclosures of Regan, J. Biol. Chem. 269: 9 (1994) (expression cloning yields DNA coding for insect diuretic hormone receptor), and Pratt et al., Biochem. Biophys. Res. Comm. 163: 1243 (1989) (an allostatin is identified in *Diploptera punctata*). See also U.S. patent No. 5,266,317 to Tomalski et al., who disclose genes encoding insect-specific, paralytic neurotoxins.

10 (G) An insect-specific venom produced in nature by a snake, a wasp, etc. For example, see Pang et al., Gene 116: 165 (1992), for disclosure of heterologous expression in plants of a gene coding for a scorpion insectotoxic peptide.

15 (H) An enzyme responsible for an hyperaccumulation of a monterpene, a sesquiterpene, a steroid, hydroxamic acid, a phenylpropanoid derivative or another non-protein molecule with insecticidal activity.

20 (I) An enzyme involved in the modification, including the post-translational modification, of a biologically active molecule; for example, a glycolytic enzyme, a proteolytic enzyme, a lipolytic enzyme, a nuclease, a cyclase, a transaminase, an esterase, a hydrolase, a phosphatase, a kinase, a phosphorylase, a polymerase, an elastase, a chitinase and a glucanase, whether natural or synthetic. See PCT application WO 93/02197 in the name of Scott et al., which discloses the nucleotide sequence of a callase gene. DNA molecules which contain chitinase-encoding sequences can

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be obtained, for example, from the ATCC under accession Nos. 39637 and 67152. See also Kramer et al., *Insect Biochem. Molec. Biol.* 23: 691 (1993), who teach the nucleotide sequence of a cDNA encoding tobacco hookworm chitinase, and Kawalleck et al., *Plant Molec. Biol.* 21: 673 (1993), who provide the nucleotide sequence of the parsley *ubi4-2* polyubiquitin gene.

5 (J) A molecule that stimulates signal transduction. For example, see the disclosure by Botella et al., *Plant Molec. Biol.* 24: 757 (1994), of nucleotide sequences for mung bean calmodulin cDNA clones, and Griess et al., *Plant Physiol.* 104: 1467 (1994), who provide the nucleotide sequence of a maize calmodulin cDNA clone.

10 (K) A hydrophobic moment peptide. See U.S. patent applications serial No. 08/168,809 (disclosure of peptide derivatives of Tachyplesin which inhibit fungal plant pathogens) and serial No. 08/179,632 (teaches synthetic antimicrobial peptides that confer disease resistance), the respective contents of which are hereby incorporated by reference.

15 (L) A membrane permease, a channel former or a channel blocker. For example, see the disclosure by Jaynes et al., *Plant Sci.* 89: 43 (1993), of heterologous expression of a cecropin- $\beta$  lytic peptide analog to render transgenic tobacco plants resistant to *Pseudomonas solanacearum*.

20 (M) A viral-invasive protein or a complex toxin derived therefrom. For example, the accumulation of viral coat proteins in transformed plant cells imparts resistance to viral infection and/or disease development effected by the virus from which the coat protein gene is derived, as well as by related viruses. See Beachy et al., *Ann. Rev.*

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5 *Phytopathol.* 28: 451 (1990). Coat protein-mediated resistance has been conferred upon transformed plants against alfalfa mosaic virus, cucumber mosaic virus, tobacco streak virus, potato virus X, potato virus Y, tobacco etch virus, tobacco rattle virus and tobacco mosaic virus. *Id.*

10 (N) An insect-specific antibody or an immunotoxin derived therefrom. Thus, an antibody targeted to a critical metabolic function in the insect gut would inactivate an affected enzyme, killing the insect. Cf. Taylor et al., Abstract #497, SEVENTH INT'L SYMPOSIUM ON MOLECULAR PLANT-MICROBE INTERACTIONS (1994) (enzymatic inactivation in transgenic tobacco via production of single-chain antibody fragments).

15 (O) A virus-specific antibody. See, for example, Tavladoraki et al., *Nature* 366: 469 (1993), who show that transgenic plants expressing recombinant antibody genes are protected from virus attack.

20 (P) A developmental-arrestive protein produced in nature by a pathogen or a parasite. Thus, 25 fungal endo  $\alpha$ -1,4-D-polygalacturonases facilitate fungal colonization and plant nutrient release by solubilizing plant cell wall homo- $\alpha$ -1,4-D-galacturonase. See Lamb et al., *Bio/Technology* 10: 1436 (1992). The cloning and characterization of a gene which 30 encodes a bean endopolygalacturonase-inhibiting protein is described by Toubart et al., *Plant J.* 2: 367 (1992).

35 (Q) A developmental-arrestive protein produced in nature by a plant. For example, Logemann et al., *Bio/Technology* 10: 305 (1992), have shown that transgenic plants expressing the barley

ribosome-inactivating gene have an increased resistance to fungal disease.

II. Genes That Confer Resistance To A Herbicide, For Example:

5 (A) A herbicide that inhibits the growing point or meristem, such as an imidazalinone or a sulfonylurea. Exemplary genes in this category code for mutant ALS and AHAS enzyme as described, for example, by Lee et al., *EMBO J.* 7: 1241 (1988), and Miki et al., *Theor. Appl. Genet.* 80: 449 (1990), respectively.

10 (B) Glyphosate (resistance imparted by mutant EPSP synthase and aroA genes, respectively) and other phosphono compounds such as glufosinate (PAT and bar genes), and pyridinoxy or phenoxy propionic acids and cyclohexones (ACCase inhibitor-encoding genes). See, for example, U.S. patent No. 4,940,835 to Shah et al., which discloses the nucleotide sequence of a form of EPSP which can confer glyphosate resistance. A DNA molecule encoding a mutant aroA gene can be obtained under ATCC accession No. 39256, and the nucleotide sequence of the mutant gene is disclosed in U.S. patent No. 4,769,061 to

15 Comai. European patent application No. 0 333 033 to Kumada et al. and U.S. patent No. 4,975,374 to Goodman et al. disclose nucleotide sequences of glutamine synthetase genes which confer resistance to herbicides such as L-phosphinothricin. The nucleotide sequence of a phosphinothricin-acetyl-transferase gene is provided in European application

20 No. 0 242 246 to Leemans et al. De Greef et al., *Bio/Technology* 7: 61 (1989),

25 describe the production of transgenic plants that express chimeric bar genes coding for phosphinothricin acetyl transferase activity. Exemplary of genes

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5 conferring resistance to phenoxy propionic acids and cyclohexones, such as sethoxydim and haloxyfop, are the *Acc1-S1*, *Acc1-S2* and *Acc1-S3* genes described by Marshall et al., *Theor. Appl. Genet.* 83: 435 (1992).

10 (C) A herbicide that inhibits photosynthesis, such as a triazine (*psbA* and *gs+* genes) and a benzonitrile (nitrilase gene). Przibilla et al., *Plant Cell* 3: 169 (1991), describe the transformation of *Chlamydomonas* with plasmids encoding mutant *psbA* genes. Nucleotide sequences for nitrilase genes are disclosed in U.S. patent No. 4,810,648 to Stalker, and DNA molecules containing these genes are available under ATCC accession Nos. 53435, 67441 and 15 67442. Cloning and expression of DNA coding for a glutathione S-transferase is described by Hayes et al., *Biochem. J.* 285: 173 (1992).

20 20 III. Genes That Confer Or Contribute To A Value-Added Trait, Such As:

(A) Nutritional enhancement, as illustrated by

25 (1) Higher lysine content: A cereal such as maize could be transformed with a gene that increases lysine content, making the cereal nutritionally more complete and thereby eliminating need for added lysine, for example, in poultry and swine feeds.

30 (2) Higher methionine content: A gene would be added to increase methionine levels in a cereal crop to offset an overall low methionine content, for example, in a poultry feed which combines lower- and higher-methionine components such as soybean and maize, respectively.

## (B) Decreased phytate content

5 (1) Introduction of a phytase-encoding gene would enhance breakdown of phytate, adding more free phosphate to the transformed cereal. For

example, see Van Hartingsveldt et al., *Gene* 127: 87 (1993), for a disclosure of the nucleotide sequence of an *Aspergillus niger* phytase gene.

10 (2) A gene could be introduced that reduces phytate content. This could be accomplished, for example, by cloning and then re-introducing DNA associated with the single allele which is responsible for maize mutants characterized by low levels of phytic acid. See Raboy et al., *Maydica* 35: 383 (1990).

15 (C) Modified carbohydrate composition effected, for example, by transforming maize with a gene coding for an enzyme that alters the branching pattern of starch. See Shiroza et al., *J. Bacteriol.* 170: 810 (1988) (nucleotide sequence of *Streptococcus mutans* fructosyltransferase gene), Steinmetz et al., *Mol. Gen. Genet.* 200: 220 (1985) (nucleotide sequence of *Bacillus subtilis* levansucrase gene), Pen et al., *Bio/Technology* 10: 292 (1992) (production of transgenic plants that express *Bacillus licheniformis*  $\alpha$ -amylase), Elliot et al., *Plant Molec. Biol.* 21: 515 (1993) (nucleotide sequences of tomato invertase genes), Søgaard et al., *J. Biol. Chem.* 268: 22480 (1993) (site-directed mutagenesis of barley amylase gene), and Fisher et al., *Plant Physiol.* 102: 1045 (1993) (maize endosperm starch branching enzyme II).

Synthesis of genes suitably employed in the present invention can be effected by means of mutually priming, long oligonucleotides. See, for example, Ausubel et al. (eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, pages 5 8.2.8 to 8.2.13 (Wiley Interscience 1990), and Wosnick et al., *Gene* 60: 115 (1987). Moreover, current techniques which employ the polymerase chain reaction permit the synthesis of genes as large as 1.8 kilobases in length. See Adang et al., *Plant Molec. Biol.* 21: 1131 (1993), and 10 Bambot et al., *PCR Methods and Applications* 2: 266 (1993).

Maize lines that can be transformed via the present invention include, among others, inbreds that are employed in producing commercial hybrids. These inbreds, 15 both proprietary and publicly-available, span many heterotic families. The preferred representatives within the heterotic groupings, and the relative use of entire heterotic patterns, vary with the market in question. For example, different germplasm is favored when breeding 20 for the continental United States, including different geographic areas of adaptation (for example, the South, the East, the West, the North and the Central Corn Belt), for Europe and for South America, as well as for other international markets.

Callus-mediated methodology is unsuitable for many inbreds which do not produce the required callus response or which provide callus that grows in a manner rendering the methodology unusably inefficient ("recalcitrant" inbreds). Accordingly, such methodology has been limited 25 to a large extent to transformation of a few genotypes, such as, in maize, A188, A188 x B73, H99, Pa91, FR16 and 30 genotypes obtained via a cross involving one of these genotypes. By contrast, meristem transformation pursuant to the present invention is applicable to any line, 35 regardless of how that line responds to callus-mediated transformation. Thus, even cereal lines heretofore deemed recalcitrant to transformation can be transformed stably via the present invention. Illustrative of the

maize inbreds thus affected are PHT47, PHP02, PHV78, PHK05, PHW20, PHR62, PHN37, PHM10, PHV37, PHJ65, PHBW8, PHK29, PHJ33, PHP60, PHN73, and PHHV4. By the same token, the present invention should be applicable to 5 newly-developed inbreds and to new heterotic groups which are created through the combination of existing germplasm, including "exotic" material brought into breeding programs from sources in the tropics and elsewhere.

10 According to a preferred embodiment, therefore, the present invention contemplates a transgenic plant that belongs to a cereal line that is recalcitrant to callus-based method transformation. Conversely, another preferred embodiment encompasses transgenic maize plants 15 that are not produced by transformation of A188, A188 x B73, H99, Pa91 or FR16. In this context, the phrase "cereal line" denotes a group of gramineous plants of the sub-family Poaoideae which display relatively little variation between individuals with respect to more than 20 one distinctive trait, generally although not exclusively by virtue of several generations of self-pollination. (In addition, the term "line" here is used sufficiently broadly to include a group of plants vegetatively propagated from a single parent plant, via tissue culture 25 techniques.) A plant is said to "belong" to a particular line if it (A) is a primary transformant ( $T_0$ ) plant regenerated from material of that line or (B) has a pedigree comprised of a  $T_0$  plant of that line. In this context, the term "pedigree" denotes the lineage of a 30 plant, e.g., in terms of the sexual crosses effected such that a gene or a combination of genes, in heterozygous (hemizygous) or homozygous condition, imparts a desired trait to the plant.

35 The present invention is further described in the following examples, which are illustrative only. In carrying out the examples, a general procedure was followed for biolistic transformation. According to this procedure, 60 mg of 1.0 to 1.8  $\mu\text{m}$  tungsten

microparticles (source: General Electric) were suspended in 2 ml of 0.1 M HNO<sub>3</sub>, and sonicated for twenty minutes on ice. After centrifugation at 10,000 rpm to remove HNO<sub>3</sub>, 1 ml of sterile deionized water was added,  
5 followed by a brief sonication and further centrifugation. This water rinse was repeated twice, after which the water was removed and 1 ml of 100% EtOH was added. The particles were resuspended by sonication, and the EtOH rinse was repeated. After the addition of  
10 1 ml of sterile, deionized water and a further sonication, four aliquots of the resulting suspension (250  $\mu$ l each) were pipetted into separate tubes (2 ml volume). Sterile, deionized water (750  $\mu$ l) was added to each tube, which then could be stored at -20°C. For  
15 purposes of DNA preparation,

50  $\mu$ l of the sonicated tungsten microparticle suspension were pipetted into a 1.5 ml tube, to which was added 1 to 10  $\mu$ g of the foreign DNA. After mixing, 50  $\mu$ l of 2.5 M CaCl<sub>2</sub> solution were added and, with further mixing, 20  $\mu$ l of 0.1 M spermidine also were introduced.  
20 The resulting composition was mixed, sonicated, and then centrifuged for about ten seconds. After the supernatant was withdrawn and 250  $\mu$ l of 100% EtOH were added, the composition again was sonicated and centrifuged, and the supernatant was withdrawn.  
25 Finally, 30  $\mu$ l of 100% EtOH solution were added to the composition, which thereafter was used, in a 5  $\mu$ l aliquot per shot, with rupture disks ranging from 200-1100 p.s.i.

Example 1. TRANSFORMATION WITH NON-LETHAL SELECTION

## (A) Evaluation of Maize Histone Promoter Linked to NPTII

5       Ears of a proprietary maize genotype, designated "N10000" for purposes of this description, were harvested seven days after pollination at the early coleoptilar stage of development. Harvested ears were surface-sterilized in 50% Chlorox with Tween 20 for 20 minutes, and then rinsed three times with sterile deionized water.

10      Kernel tops were removed with a scalpel and embryos were dissected from endosperm. Sixty-seven embryos were placed axis side up, 10 embryos per plate, onto maturation medium (MS salts, 0.1 g/L myoinositol, MS vitamins, 0.5 mg/L zeatin, 150 g/L sucrose, and 6 g/L

15      Sea-Kem agarose; pH 5.6 prior to autoclaving). Embryos were incubated overnight at 28°C in the dark before bombardment.

20      In these studies, embryos were transformed with plasmids DP6212 and DP3953. DP6212 contains the 2xhistone-143 promoter, the first intron of the maize *ADH1* gene, the *nptII* gene encoding neomycin phosphotransferase (NPTII), and a 3' transcript processing region from the Proteinase Inhibitor II (*PinII*) gene of potato. DP3953 contains the ubiquitin promoter, the first intron of the *ubi* gene, the gene encoding  $\beta$ -glucuronidase (GUS), and a 3' transcript processing region from the *PinII* gene. Embryos were bombarded with DP6212 and DP3953, mixed at a 1:1 ratio, at a concentration of one 1  $\mu$ g of total DNA per tube of acid-washed tungsten particles. This concentration, at ten times less than the standard, was optimal for yielding transformants with more uniform GUS staining patterns and yet having no detrimental effect on the function of the selectable marker or the frequency of transformation.

35      Pursuant to the above-discussed biolistics protocol, the particles were delivered as five 5- $\mu$ l shots per tungsten tube, using a PDS-1000 Helium gun with 1100

p.s.i. rupture disks. All embryos received one bombardment per plate.

After bombardment, embryos were maintained at 28°C in the dark for seven days on Maturation Medium. Embryos 5 then were transferred to 272K shoot elongation medium (MS salts, 0.1 g/L myoinositol, MS vitamins, 30g/L sucrose, and 4g/L gelrite) which contained 150 mg/L tobramycin sulfate as the selection agent. Embryos were incubated in the light at 28°C. At the time of transfer, embryos 10 had elongated cotyledons.

At two, three and four weeks after bombardment, recovered plantlets were sampled and analyzed for GUS expression via methodology described by McCabe et al., *Bio/Technology* 87: 923-26 (1988). Leaf tips were placed 15 in about 200 µl of histochemical stain and allowed to incubate at 37°C overnight in the dark to maximize GUS expression. Data from the first and second leaves are summarized in the following table.

Table 1

20 *GUS Activity in Tissues of Transformed N10000*

<u>Plate</u>	<u>#Plants Analyzed</u>	<u>#GUS +</u>	<u>GUS Sector Type</u>
1	9	6	half leaves
2	8	5	half leaves or spotty
25	3	4	linear sectors
	4	6	half leaves or spotty
	5	7	linear sectors
	6	11	complete staining

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Any plantlets showing positive sectors were transferred to culture tubes containing shoot elongation medium that did not contain tobramycin. Each new leaf was examined for GUS expression. Plantlets that were 35 consistently positive for GUS were transferred to the greenhouse for maturation when root development was well established. Plants that stopped expressing GUS were observed for phenotype changes, i.e., necrosis, bleaching

or general lack of growth, which indicated that an escape from selection had occurred. Plants with normal phenotypes were analyzed for NPTII protein by means of a NPTII ELISA kit available from 5'-->3', Inc., 5603 5 Arapahoe Road, Boulder, CO 80303 (catalog No. 5307-661-514). Positives were transferred to the greenhouse.

Transgenic plants maturing in the greenhouse were sampled for GUS activity or NPTII protein in each new leaf and in tassel and ear tissues to characterize the 10 expression pattern in each plant. Pollinations were completed as selfs or as sibs. Eight to ten days after pollination, embryos were rescued by harvesting and surface sterilizing the ears, excising the embryos and placing the embryos on shoot elongation medium for 15 germination. (This procedure was not required but was preformed to accelerate the analysis process.) T1 leaf tissue was sampled for GUS histochemical assays and painted with 2% kanamycin sulfate in 0.2% SDS buffer to verify transmission of the transgenes. Samples of mature 20 leaves were harvested from the T0 transformant for Southern analysis to further characterize the transformation.

Histochemical analysis of one N10000 plant, designated "2-4," demonstrated that GUS was expressed in 25 leaves, silks (primary ear), primary ear husks and cob, and central spike and branches of tassel. In addition, Southern analysis confirmed the presence of the NPTII structural gene in leaf tissue harvested from the mature R<sub>0</sub> plant. Segregation of this hybridizing band 30 correlated with NPTII-positive ELISA results in this plant. Analysis of the central stalk also showed GUS expression in epidermal layer of adventitious roots and in a vascular bundle of the central stalk.

(B) *Transformation of Maize Lines with Non-Lethal Selection*

Experiments were performed to determine the efficacy of non-lethal selection in a variety of maize genotypes. Genotype N10000 and several other proprietary genotypes, designated "P10000," "W20000," "E10000," "PHP02" and "R20000," respectively, for purposes of this description, were transformed with plasmids DP6212 and DP3953 as described above. Table 2 below enumerates data showing that the non-lethal selection method is applicable to various maize lines, based on co-transformation experiments where the expression of the second nonselected gene was used to assess stable sector frequency and size.

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Table 2

*Percentage of Plantlets Expressing GUS Activity After Germination on Nonlethal Selection Medium*

	<u>Experiment</u>	<u>N10000</u>	<u>P10000</u>	<u>W20000</u>	<u>E10000</u>	<u>PHP02</u>	<u>R20000</u>
20	A	65.6	---	---	---	---	---
	B	23.1	---	---	---	---	---
	C	---	---	---	16.7	---	---
	D	---	---	---	71.1	---	---
	E	00.0	---	38.5	---	---	---
	F	16.4	---	---	---	7.3	---
25	G	12.3	---	---	---	---	20.0
	H	39.1	---	---	---	---	---
	I	---	---	---	20.0	---	15.5
	J	---	---	---	7.2	---	---
	K	---	---	---	4.3	---	---
30	L	---	5.0	---	---	---	---
	M	---	---	---	5.0	---	1.3
	<u>Averages</u>	26.1	5.0	38.5	20.7	7.3	12.3

(C) *Evaluation of Transformation Frequency  
When Meristem Reorganization Is Effected  
by Mechanical Disruption of Apical Dome  
Prior to Bombardment*

5       Ears of genotypes E10000 and W20000 were harvested at the early coleoptilar stage of development and at 11 and 9 days, respectively, after pollination. One hundred and sixty embryos were isolated and incubated on maturation medium, as described above.

10      The apical dome of several embryos was disrupted prior to bombardment to force the meristem to reorganize and form new meristematic areas. Mechanical disruption was performed by means of micromanipulation needles, ranging from 0.5  $\mu\text{m}$  to 5  $\mu\text{m}$  15 in diameter, which were attached to a World Precision Instruments M3301 micromanipulator. Needle penetration of each embryo was effected in the center of the apical dome to a depth ranging from a few microns to a few hundred microns, depending on the morphology of the 20 embryo (embryos with a larger scutellum will tolerate deeper penetration). The preferred targeted depth of penetration was between 50  $\mu\text{m}$  and 150  $\mu\text{m}$ . Embryos then were bombarded with NPTII and GUS constructs, as described above.

25      Embryos were maintained in the dark at 28°C for seven days on Maturation Medium and then transferred to 272K medium containing 150 mg/L Tobramycin sulfate. At time of transfer, embryos had multiple meristem formation with elongated cotyledons. Embryos were 30 incubated in the light at 28°C.

35      At two, three and four weeks post-bombardment, first and second leaves of recovered plantlets were analyzed for GUS expression by histochemical assay. Table 3 shows the results of GUS assays, as well as observations on meristem formation.

Table 3*Effect of Mechanical Disruption on Transformation Efficiency and Meristem Formation*

	<u>Manipulation</u>	<u>N</u>	<u># New Meristems</u>	<u>% GUS-Positive Plants</u>
5	150 $\mu$ M	80	89	45.9% [17/37 analyzed]
	none	60	0	37.5% [3/8 analyzed]

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These data demonstrate that the mechanical disruption of the apical dome resulted in new meristem formation and a higher frequency of transformation. In addition, mechanical disruption provided a more continuous GUS expression pattern in relation to non-manipulated plants, which displayed a narrower, more spotty pattern of GUS expression. Thus, meristems not disrupted frequently exhibited leaf tip GUS expression only, whereas most of the meristems that were disrupted showed wide, continuous sectors in leaves.

Example 2. TRANSFORMATION WITH SHOOT MULTIPLICATION(A) General Methodology

Embryos at the coleoptilar stage were isolated and cultured scutellum side down on an embryo 25 maturation medium (10-20 embryos/plate). Since there can be considerable seasonal and genotypic variation affecting embryo ontogeny, embryo stage rather than size or days after pollination, was monitored carefully.

30 Embryos typically were matured on MS-based medium containing 0.5 mg/L zeatin, 1 mg/L indoleacetic acid, and elevated sugar levels which serve as an osmoticum. The embryos were cultured for a period ranging from 0-48 hours post-isolation, with 12-24 hours being optimal. Meristems then were bombarded

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with genes conferring kanamycin or streptomycin resistance, along with other nonselected genes, such as agronomic or visual marker genes.

After bombardment, the embryos were cultured in the dark to promote germination. After one to two weeks, the embryos were moved to a germination medium, such as hormone-free or low-hormone MS medium. The germinated plantlets generally had a swelling at the junction between the mesocotyl and epicotyl. This swelling occurred in the region containing the developing shoot meristem.

Two to three millimeter sections including the meristem were excised and cultured on a shoot proliferation medium which contained the appropriate hormones and a selection agent. The sections were regularly trimmed of elongated leaves and transferred to fresh medium every 10 to 14 days. Cultured meristems were incubated at 28°C in the dark. After three to nine weeks, the proliferating meristems were transferred to an illuminated culture room.

Transformed sectors were identified one to two weeks after culture in the light, based on their green phenotype, i.e., nontransformed tissue remained bleached upon selection. In general, plants were regenerated by lowering the hormone concentration, although in some genotypes cytokinin concentrations were increased to promote plant regeneration. Since regenerated plants sometimes have difficulty rooting, rooting was promoted by culture on SH medium with 1 mg/l NAA, or by nicking the base of the stem and dipping the shoots in a 1 mg/ml NAA solution.

(B) *NPTII Transformation of Honey and Pearl*

One hundred eighty coleoptilar-stage maize embryos of the Honey and Pearl variety were harvested nine days after pollination. The scutella of the isolated embryos averaged 0.48 mm in length. These embryos were placed on embryo Maturation Medium (10

embryos per plate) and cultured overnight in the dark at 28°C.

Sixteen plates of the embryos were bombarded twice according to the above-described method with 5 plasmid DP551, using 1.8  $\mu$ m tungsten particles at a DNA concentration of 10  $\mu$ g DNA/tube of tungsten. Plasmid DP551 contains ADH intron 1, GUS gene, and nos terminator, as well as ADH intron 1, NPTII gene, PinII terminator. Both GUS and NPTII genes are regulated by 10 35S CaMV sequences. Plates containing these embryos were cultured and matured in the dark at 28°C. Eight days later, a few of the embryos were placed in X-Gluc histochemical stain. All embryos contained intense blue staining, indicating GUS activity.

15 Most of the embryos had germinated nineteen days after particle bombardment. At this time, the region containing the meristem and leaf primordia was excised as described above and cultured on agar solidified MS medium with 2 mg/L BAP and 50 mg/L kanamycin. Leaf 20 tissue was stained and chimeric blue staining sectors were observed in eight of the sixteen plates. The region containing the meristem was trimmed of elongated leaves and transferred to fresh medium every 10 to 14 days. Twenty six days after bombardment, the level of 25 kanamycin was increased to 100 mg/L. Proliferating meristems were transferred to the light a week later. These experiments produced three independent transformation events. Two of the transformants have been characterized by PCR, GUS staining, NPTII ELISA 30 assay and Southern analysis. One of these events exhibited strong GUS activity and high levels of NPTII protein. The T1 and T2 generations from this event were used for subsequent analysis. Progeny displayed a 35 co-segregating, 1-to-1 ratio after outcrossing, based on both GUS activity and NPTII ELISA results (see Table 4) consistent with Mendelian inheritance of the integrated genes. Integration and segregation of the NPTII gene, which correlated with positive NPTII ELISA

results, were demonstrated through Southern analysis of T<sub>1</sub> plants.

(C) *aadA* Transformation of Honey and Pearl

Coleoptilar stage Honey and Pearl embryos were 5 isolated and cultured on 288B medium (MS medium with 0.5 mg/l zeatin, 1mg/l IAA, 0.25M sorbitol, and 4% sucrose solidified with 3 g/l gelrite). Eight plates with ten embryos per plate were bombarded once, as described above. Each particle preparation (enough for 10 six shots) employed a combined total of 10 µg of DNA (5 µg DP4790 + 5 µg DP460 or DP3536). Plates 1 to 4 were bombarded with plasmid DP4790, which contains a 35S CaMV promoter, omega', *aadA* and *ocs* terminator (provided by Dr. Jonathan Jones, John Innes Institute), 15 and with plasmid DP460, which contains a 35S CaMV promoter, *ADH* intron, *GUS* gene, and *nos* terminator. Plates 5 to 8 were bombarded with plasmids DP4790 and DP3536. The latter plasmid contains a *cab* promoter, *ADH* intron 6, *GUS* gene, and *ocs* terminator. All 20 embryos were grown and germinated as described in part (B) of this example, *supra*. After germination, the regions containing the meristems were cultured on agar-solidified MS medium containing 2 mg/L BAP and 100 mg/L streptomycin sulfate.

25 After cultured meristems were moved to an illuminated culture room, a green sector was observed on a proliferating meristem on plate 6. All other cultured meristems were white due to streptomycin bleaching. GUS staining at this time revealed a mix of 30 sectored and non-sectored blue staining leaves. About seven weeks after bombardment, sorting out was observed in the leaves from the transformation event on plate six. Some leaves were non-sectored GUS+ while others were still mericinal. Transformation was 35 confirmed using PCR, GUS staining and Southern analysis.

(D) Transformation of an elite inbred

Eight days after pollination, coleoptilar stage embryos of an elite inbred, designated "B30000" for purposes of this description, were isolated and 5 cultured on 288L medium in fifteen plates containing twenty embryos per plate. Twelve plates were bombarded, using standard protocols. Briefly, particle bombardment was performed with six shots using 650 psi rupture disks and 1  $\mu$ m tungsten particles, which were 10 coated with plasmids DP5397 (proprietary agronomic gene) and DP5606 (Ubi promoter/Ubi-intron/ NPTII/pin II terminator linked to cab promoter/ADH intron 6/GUS/ocs terminator) at a concentration of 5  $\mu$ g DNA/particle preparation tube for each plasmid.

15 Plasmid DP5397 is a proprietary agronomic plasmid which contains a *Bt* gene, while plasmid DP5606 contains the Ubi promoter, Ubi intron, *NPTII* gene, and *PinII* terminator, which is linked to a cab promoter, *ADH* intron 6, *GUS* gene, and *ocs* terminator.

20 After bombardment the meristems were cultured on agar solidified MS medium containing 2 mg/L BAP, 0.25 mg/L, 2,4-dichlorophenoxy acetic acid and 3% sucrose. Five weeks after bombardment, meristems were placed on kanamycin selection (100 mg/L). To avoid 25 irreversible bleaching of the meristems, this tissue was cycled between selective and non-selective media.

30 Five months after bombardment, a large green sector was removed from a bleached shoot culture. Three small leaves were removed from the sector and stained with X-Gluc. The leaves were found to express GUS activity in non-epidermal cells.

35 A single plant was regenerated from this series of experiments. The plant produced copious amounts of pollen and several ears. The pollen was found to be segregating for GUS expression, which was surprising since this gene was under the control of the cab promoter. All leaves of this plant exhibited strong, non-sectored GUS activity. The tassel glumes also were

positive for GUS activity. Samples of leaf tissue from this T<sub>0</sub> plant contained the NPT-II and Bt proteins (as verified by their respective ELISA's) and exhibited strong GUS activity (fluorometric analysis). The GUS histochemical assay verified transmission to progeny in 42 of 106 seedlings sampled to date, which is consistent with Mendelian inheritance.

**Example 3. TRANSFORMATION REGIMEN EMPLOYING IMMATURE EAR AND TASSEL MERISTEMS**

10           ***(A) Excision of immature ears***

From plants harvested seven to nine weeks after planting, leaves were removed aseptically, one at a time, and the ears were exposed. The ears were dissected out of the husks under a dissecting microscope. Longitudinal bisection of the ears increased the response and exposed the meristems more fully to bombardment.

15           ***(B) Staging and selection of responsive explants***

The size of the whole excised ear and the developmental stage of the meristems were found to be reliable indicators of proper timing of harvest. Smaller ears are less developmentally determined and more responsive to hormonal stimuli, but fewer meristems survive resulting in fewer targets for transformation. Although smaller inflorescences have been used, two millimeters was used as the practical lower size limit for transformation experiments. The upper limit for selection of responsive targets was determined by meristem stage; developmental plasticity decreased dramatically once the glumes began to be obvious and approached the sides of the meristematic dome.

20           ***(C) Initial culture medium***

Various media have been used, and inbreds respond differently to these variations. A preferred medium used in the initial stage of floral meristem culture (used for various genotypes) consisted of Murashige and Skoog salts, MS vitamins, 0.1 mg/l 2,4-D, 0.5 gm/l 6-

BAP, 1-proline at 12.2  $\mu$ M, 8% sucrose, and silver nitrate at 30 mg/l. A preferred gelling agent is GELRITE (product of Merck and Co, Inc./Kelco division, Rahway, NJ) at 3.5 g/l.

5                   (D) Bombardment

Immature ear explants were bombarded using 650 psi rupture disks and a stainless steel screen (100  $\mu$ m mesh size) suspended approximately 0.5 to 1.0 cm above the tissue. DNA precipitation and other bombardment

10 parameters were as described in Example 1.

(E) Subsection, subculture and selection

Maintenance of rapid growth and survival of individual meristems was achieved by subsecting the ears four to six days after isolation, into pieces with four to eight meristems each. These pieces were cultured onto shoot multiplication medium, which has the same basal composition as the initial culture medium (above) but with 1 mg/l BAP and 3% sucrose. Meristem tissue was subcultured repeatedly, at two week

20 intervals on the shoot multiplication medium.

Incubation of bombarded ear meristems in X-gluc consistently resulted in high frequencies of transient GUS expression two days after bombardment. Stable sectors in leaves produced by multiple shoot clumps were found to express GUS one month after bombardment. At this stage, leaves were approximately 1 to 2 cm in length, and transformed sectors were found that extended more than half the length of the leaf. In addition, one meristem sacrificed at this stage

30 expressed high levels of GUS in a histochemical assay.

One month of shoot multiplication was followed by one month of selection using 100 mg/l streptomycin. After this treatment, all material was subcultured once more onto medium without the selective agent, and were 35 additionally moved into the light. Leaves and shoots in non-selected cultures quickly turned green. Leaves in selected cultures remained bleached (white).

(F) Plant regeneration

Putatively transformed shoots clumps were transferred to medium lacking plant growth regulators. Varying degrees of leaf development occurred on 1 mg/l 5 BAP, and shoots soon formed and elongated in the absence of hormones.

(G) Rooting

Rooting at high frequency was effected via several days of exposure to MS- or SH-based media with 1-5 mg/l 10 NAA.

Example 4. TRANSFORMATION OF EARLY PROEMBRYO, MID PROEMBRYO, LATE PROEMBRYO, TRANSITIONAL AND EARLY COLEOPTILAR-STAGE EMBRYOS

Immature embryos at the mid proembryo, late 15 proembryo, transitional and early coleoptilar stage were harvested and cultured on culture medium 610A, containing high concentrations of cytokinin and osmoticum. The 610A culture medium comprised MS salts, MS vitamins, 100 mg/L myo-inositol, 0.4 mg/L thiamine-HCl, 1 mg/L zeatin riboside, 0.1 mg/L BAP, 60 g/L sucrose, 400 mg/L asparagine, and 7 g/L Hazelton TC agar. After one day of recovery, the embryos were 20 bombarded with DNA, by means of the particle gun as described above, and punctured in the center of the 25 area of where the apical meristem will develop with a 0.5 $\mu$ m micromanipulation needle.

Embryos were allowed to mature for 7 days in the 30 dark and then transferred to a hormone-free medium containing 1 mg/l bialaphos. Following another 7 days of culture on hormone-free medium in the dark, the 35 embryos were transferred to germination medium, and cultured in the light for continued germination. As leaves developed, plant phenotype was observed and samples were taken to check for sector formation by histochemical assay (GUS) as described above.

Healthy plants with normal phenotype and/or reporter gene activity were transferred to the

greenhouse for maturation. The data shown in Table 4, which were generated via the above-discussed protocol, demonstrate for inbred N10000 the sector frequency obtained across several similar experiments, using 5 embryos staged at mid proembryo, late proembryo, transitional, and early coleoptilar.

Table 4

10 *Sector Frequency Obtained With  
Mid Proembryo, Late Proembryo, Transitional and  
Early Coleoptilar Stage Embryos*

Embryo Stage	N	Transgenes	GUS frequency	GUS pattern of expression	Sector Placement
Early Coleoptilar	328	BAR/GUS	14.2%	Leaf tips and files of 1-3 cells	Leaves 1 and 2
Transitional	250	BAR/GUS	22.5%	Saddle and Linear	Sectors start at Leaf 1, 5, or 11
Late Proembryo	200	BAR/GUS	34%	Saddle and Linear	Sectors start at Leaf 1 or Leaf 5
Mid Proembryo	110	BAR/GUS	3%	Linear and whole leaf	Sectors start at Leaf 1

20 The GUS frequency observed after targeting mid proembryos reflected, at the time these data were collected, a relatively poor survival rate after bombardment and selection. But the addition of 1 mg/l zeatin to medium 610A, an increasing of the agar concentration (12 g/l), and the use of lower rupture 25 disc pressure (200 p.s.i.) during particle delivery increases survival of mid proembryos after isolation and DNA delivery.

Example 5. MERISTEM TRANSFORMATION -- DIRECT  
GERMINATION APPROACH

Genotype N10000 plants were pollinated and, eight days later, embryos were placed into culture. The 5 harvested embryos thus were late-proembryo stage.

More specifically, embryos were cultured at day 0, axis up, onto modified 610A medium, containing 150 g/l sucrose, 1 mg/l zatin, and 12 g/l agar, and incubated at 28°C overnight in the dark. At day 1, following the 10 overnight incubation, the apical meristems of all embryos were disrupted in the center of the apical dome using a 0.5μm Femtotip micromanipulation needle. All embryos were returned for an overnight incubation at 28C in the dark. At day 2 bombardment was effected 15 with the PDS-1000 Helium particle gun, one shot per plate, using 650PSI rupture disks. DNA employed in this regard was DP3528+DP3953 [2x35S::BAR+UBI::GUS] at 1 μg/tube of 1-μm tungsten. At day 2 all embryos were maintained in the dark at 28°C for 7 days on 610A 20 medium to allow meristem maturation to occur. At day 7 (after 7 days on 610A), embryos were transferred to 612 medium containing MS salts and vitamins, 0.001 mg/l kinetin, 0.1 mg/l adenine sulfate, 20 g/l sucrose, 6 g/l agar and 0.5 mg/L bialaphos, for germination and 25 selection. At day 14 embryos were kept in the dark at 28°C for 7 days before transfer to the light for further germination. On days 21-49 GUS histochemical assays on developing leaves were conducted, and on day 35 growing plantlets were transferred to tubes 30 containing MS medium with no hormones and 5 mg/L bialaphos. On day 56 plant 6-1 (SID 180741) and plant 2-7 (SID 180742) were transferred to the greenhouse.

The total number of embryos cultured and bombarded 35 was 48, of which 37 developed normally. The number of embryos that grew beyond leaf 1 was 17, with four plants showing GUS expression. Two plants survived 5 mg/L-bialaphos selection with normal root

development, and were transferred to the greenhouse for maturation.

SID 180741 and 180742 both showed GUS expression at the time of greenhouse transfer and had normal leaf and root development, whereas all other plants died. SID 180742 showed GUS expression in leaves 1-8 only.

Table 5

**Sector Placement by GUS histochemical assay of SID180741**

<u>Structure</u>	<u>GUS phenotype</u>
Leaf 1	negative
Leaf 2	leaf tip
Leaf 3	leaf tip
15 Leaf 4	saddle sector: margin and midrib
Leaf 5	midrib sector
Leaf 6	saddle sector margin and midrib
Leaf 7	midrib sector
20 Leaf 8	saddle sector margin and midrib
Leaf 9	midrib sector
Leaf 10	saddle sector margin and midrib
Leaf 11	midrib sector
Leaf 12	saddle sector margin and midrib
Leaf 13	midrib sector
25 Leaf 14	half leaf
Leaf 15	half leaf
Leaf 16	half leaf
Leaf 17	half leaf
30 Leaf 18	half leaf
Leaf 19	half leaf
Leaf 20	entire leaf
Leaf 21	entire leaf
Tassel	To date: central stalk shows pollen staining; 5 tassel branches also positive
35 Anthers	Endothecium and epidermis positive
Anther glumes	Epidermis positive

Leaves were painted with 1% Ignite in lanolin paste at the V6-V8 stage of development. SID 180741 showed resistance to Ignite in the sectored (GUS expressing) areas only. SID 180742 showed no resistance to Ignite. PCR analyses were done on sampled leaves and confirmed presence of both GUS and BAR genes in SID 180741 and GUS genes in SID 180742.

One of the first major differences observed after moving towards an earlier developmental stage, i.e., when targeting late proembryos, was the production of saddle sectors (see Poethig (1986), *supra*, for a 5 description). Extant information concerning meristem organization suggested to the present inventors that this might lead to germline transmission through the tassel. Saddle sectors extend from the leaf primordium up through the central portion of the apical dome and 10 back into another portion of the leaf primordium. The extension of transgenic sectors into the central portion of the meristem greatly increases the probability of the sector contributing to the tassel and, ultimately, to pollen.

15 Previous research on maize anatomy and clonal analysis has shown that maize contains an organized, layered apical meristem beginning at the transitional stage of development. See Randolph, *J. Agric. Res.* 53: 881-916 (1936), and Poethig (1986), *supra*.

20 Furthermore, a paper by Dawe and Freeling, *Developmental Biol.* 142: 233-45 (1990), regarding cell lineages in the male flower of maize, indicated that the L1 and L2 layers of the apical meristem give rise to the two layers of the anther wall. Only the inner 25 layer is derived from the same cell lineage as the male germ cells, the L2. It also was found that events that occurred (by irradiation) before the organization of the shoot apical meristem contained sectors in both layers of the anther wall with inheritance through the 30 pollen. Events that occurred after the transitional stage of development were limited to only one cell lineage with inheritance only when sectors occurred in the L2 layer.

35 Transformant 180741 was bombarded by the particle gun at the late proembryo stage of development, before meristem layer organization occurs. It contained a saddle sector which, by definition, is a sector which traverses the apical dome and bisects the meristem, in

a region of the meristem that will later develop into the tassel (see Poethig (1986), *supra*). It also was wounded by a micro-manipulation needle, to encourage meristem reorganization, and exposed to bialaphos as the selective agent. GUS histochemical data showed expression in both layers of the leaves, in the anther wall, and in about 50% of the pollen from the central stalk.

Example 6. STABILIZATION OF TRANSGENIC SECTORS BY  
10 MEANS OF TILLERING

As noted above, tillering of transformed plants is an alternative to shoot multiplication for stabilization of transgenic sectors. Accordingly, elite lines may be induced to tiller, pursuant to the present invention, thereby stabilizing transgenic sectors.

In this example, tillering was induced in control plants using the method described by De Wolff, *Euphytica* 20: 524-26 (1971). A triangular incision was made with a number 11 scalpel blade at the approximate height of, or slightly above, the shoot apex of two week-old seedlings. The incision was made perpendicular to the plane of the leaves in order to avoid damage to the midribs. The shoot apex was removed from P10000, PHP02, G30000 and E10000 seedlings. Each of these genotypes represents inbreds from significantly different heterotic families. Untreated plants of the same genotype were used as controls. If the incision was too far above the apex the procedure was repeated just below the initial incision.

The wounded plants and untreated controls were maintained in 24 hour continuous light (greenhouse during the day, growth chamber by night) for two weeks. A replicate treatment was grown under light/dark conditions.

Significant tillering was observed in the plants from which apices were removed. The influence of continuous light on tillering frequency, relative to normal light/dark conditions, was variable and may 5 depend on genotype. The untreated controls did not tiller.

The hole made by the incision could have been plugged with lanolin and phytohormones, such as TIBA (1 mg/L) or BAP (10 mg/L), to increase tillering 10 frequency. In the alternative, or in addition to the phytohormones, selective agents such as kanamycin could have been added to the incision to identify and select transgenic sectors.

What Is Claimed Is:

1. A method for producing transgenic cereal plants that will transmit introduced DNA to progeny, comprising the steps of

5 (A) introducing foreign DNA into target cells selected from the group consisting of (i) cells of a meristem that is not enclosed by sheathing leaves and (ii) cells fated to contribute to said meristem; then  
(B) inducing reorganization of said meristem to  
10 increase transgenic sector size, whereby the likelihood that a transgenic sector will contribute to germline transmission is increased; and thereafter

15 (C) exposing said meristem to conditions under which it differentiates to form a plantlet, wherein said plantlet contains said transgenic sector or is homogeneously transformed by said foreign DNA, such that said plantlet can be grown into a transformed cereal plant that will transmit said foreign DNA to progeny.

20 2. A method according to claim 1, wherein step (A) is effected at early proembryo, mid proembryo, late proembryo, transitional or early coleoptilar stage.

25 3. A method according to claim 1, wherein said foreign DNA is introduced into a plurality of meristems, and at least some of said meristems differentiate in step (C) to form a plurality of plantlets.

30 4. A method according to claim 1, wherein said reorganization is effected through at least one manipulation selected from the group consisting of (i) imposition of a nonlethal selective pressure on said meristems, (ii) mechanically-induced meristem reorganization, and (iii) hormonally-induced shoot multiplication.

35 5. A method according to claim 1, wherein said conditions in step (C) are such that said meristems undergo maturation and plant differentiation to form

shoot apices, and wherein said method further comprises effecting reorganization of meristem tissue in said shoot apices to enlarge transformed sectors or to produce periclinal L2 chimeras.

5 6. A method according to claim 5, wherein said effecting of reorganization comprises exposing said shoot apices to nonlethal selection pressure such that transformed cells have a competitive growth advantage over nontransformed cells in said shoot apices, and the  
10 proportion of transformed cells in said shoot apices is increased.

15 7. A method according to claim 1, where step (A) comprises biolistic bombardment of an embryo at a developmental stage no later than the coleoptilar stage.

8. A method according to claim 5, wherein said effecting of reorganization comprises culturing said shoot apices under conditions such that multiple shoots proliferate therefrom through organogenesis.

20 9. A method according to claim 1, further comprising a step before step (C) of wounding said apical dome selectively.

10. A method according to claim 9, wherein said wounding is carried out before step (A).

25 11. A method according to claim 1, wherein step (A) comprises biolistic bombardment of meristems on immature ear or tassel explants.

12. A method according to claim 1, wherein said target cells are maize, sorghum, wheat, barley, oat or  
30 rice target cells.

13. A method according to claim 12, wherein said target cells are maize target cells.

35 14. A method according to claim 5, further comprising the steps of (i) dissecting out of an axillary bud from above the base of a leaf of a plantlet when a chimeric sector is observed in a substantial portion of said leaf, and then

(ii) germinating said axillary bud into a whole plant or subjecting said axillary bud to shoot multiplication.

15. A method according to claim 1, further comprising the steps of (i) removing the shoot apex from seedlings of said plantlet, whereby a wound is produced, then (ii) growing said plantlets to induce formation of a plurality of tillers, and (iii) selecting a transgenic tiller from said plurality.

10 16. A method according to claim 15, wherein phytohormones that increase tillering are introduced, subsequent to step (i), into said wound.

15 17. A method according to claim 15, wherein a selective agent is introduced after step (i) into the wound, to identify and select transgenic sectors.

18. A transgenic cereal plant that is the product of a process according to claim 1, wherein said cereal plant transmits introduced DNA to progeny and belongs to a cereal line that is recalcitrant to callus-based transformation.

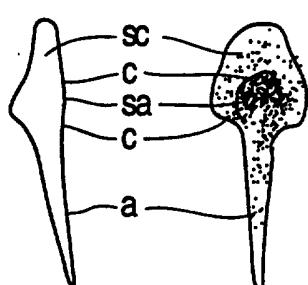
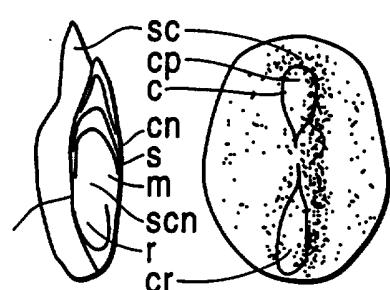
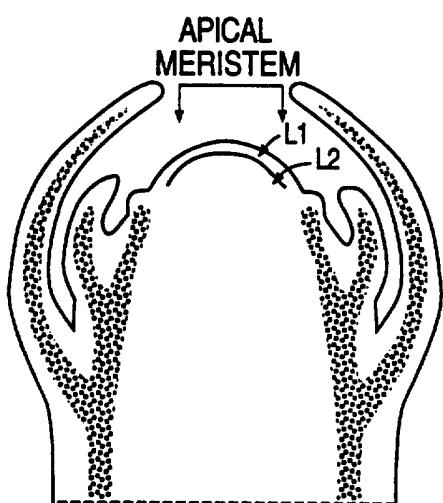
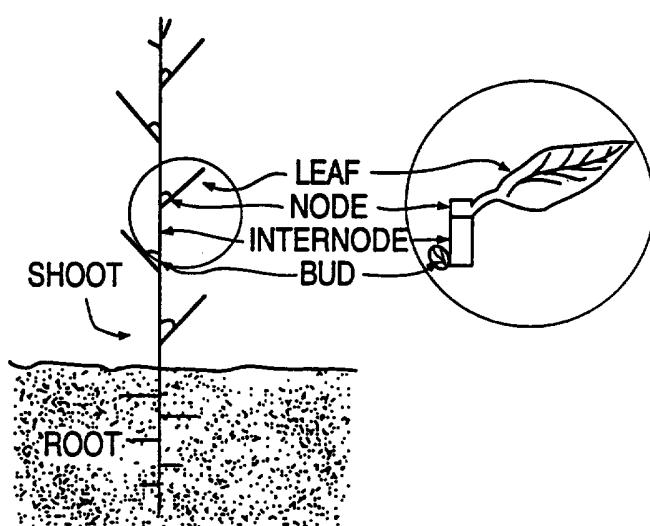
20 19. A transgenic cereal plant according to claim 18, wherein said cereal plant is a maize plant that is not produced by transformation of a genotype selected from the group consisting of A188, A188 x B73, H99, Pa91, FR16, and a genotype obtained from a cross involving any of the foregoing.

25 20. A transgenic cereal plant that is the product of a process according to claim 15, wherein said cereal plant transmits introduced DNA to progeny and belongs to a cereal line that is recalcitrant to callus-based transformation.

30 21. A transgenic cereal plant according to claim 20, wherein said cereal plant is a maize plant that is not produced by transformation of a genotype selected from the group consisting of A188, A188 x B73, H99, Pa91, FR16, and a genotype obtained from a cross involving any of the foregoing

22. A maize plant that transmits introduced DNA to progeny and that has a pedigree including a line selected from the group consisting of PHT47, PHP02, PHV78, PHK05, PHW20, PHR62, PHN37, PHM10, PHV37, PHJ65, 5 PHBW8, PHK29, PHJ33, PHP60, PHN73 and PHHV4.

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**FIG. 1a****FIG. 1b****FIG. 1c****FIG. 1d**

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FIG. 2

