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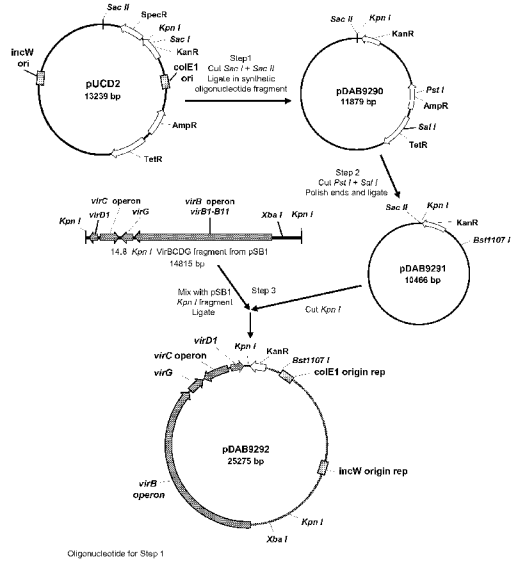

Fig. 1

(57) Abstract: *Agrobacterium* strains that harbor transformation-enhancing genes on a plasmid capable of replication independently of the *Agrobacterium* chromosome, the Ti plasmid, and plant transformation binary vectors, and uses for these *Agrobacterium* strains are provided. Additionally, *Agrobacterium* strains that are deficient in DNA recombination functions that result in instability or rearrangement of plant transformation binary vectors, and that harbor transformation-enhancing genes on a plasmid capable of replication independently of the *Agrobacterium* chromosome, the Ti plasmid, and plant transformation binary vectors, and uses for these strains, are also provided. Further included are *Agrobacterium* strains that harbor transformation-enhancing genes integrated into the *Agrobacterium* chromosome at a locus that does not interfere with or otherwise compromise the normal growth and plant transformation ability of the *Agrobacterium* cells, and uses for these *Agrobacterium* strains. Plants made using these *Agrobacterium* strains are also described.

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STRAINS OF AGROBACTERIUM MODIFIED TO INCREASE PLANT TRANSFORMATION FREQUENCY

This application claims the benefit of U.S. Provisional Application No. 61/368,965, filed July 29, 2010. The disclosure of the prior application is considered part of the disclosure of this application.

BACKGROUND

Plant transformation generally encompasses the methodologies required and utilized for the introduction of a plant-expressible foreign gene into plant cells, such that fertile progeny plants may be obtained which stably maintain and express the foreign gene. Numerous members of the monocotyledonous and dicotyledonous classifications have been transformed. Transgenic agronomic crops, as well as fruits and vegetables, are of commercial interest. Such crops include but are not limited to maize, rice, soybeans, canola, sunflower, alfalfa, sorghum, wheat, cotton, peanuts, tomatoes, potatoes, and the like. Despite the development of plant transformation systems for introducing plant-expressible foreign genes into plant cells, additional improvements which allow for increased transformation efficiency are desirable and provide significant advantages in overcoming operational disadvantages when transforming plants with foreign genes.

Several techniques are known for introducing foreign genetic material into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include acceleration of genetic material coated onto microparticles directly into cells (*e.g.,* U.S. Patent No. 4,945,050 and U.S. Patent No. 5,141,131). Other transformation technology includes silicon carbide or WHISKERS™ technology. See, *e.g.,* U.S. Patent No. 5,302,523 and U.S. Patent No. 5,464,765. Electroporation technology has also been used to transform plants. See, *e.g.,* WO 87/06614, U.S. Patent No. 5,472,869, U.S. Patent No. 5,384,253, WO 92/09696, and WO 93/21335. Additionally, fusion of plant protoplasts with liposomes containing the DNA to be delivered, direct injection of the DNA, as well as other possible methods, may be employed.

Once the inserted DNA has been integrated into the plant genome, it is usually relatively stable throughout subsequent generations. The transformed cells grow inside the

plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and may be crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties, for example, the ability to control the feeding of plant pest insects.

A number of alternative techniques can also be used for inserting DNA into a host plant cell. Those techniques include, but are not limited to, transformation with T-DNA delivered by *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as the transformation agent. Plants may be transformed using *Agrobacterium* technology, as described, for example, in U.S. Patent No. 5,177,010, U.S. Patent No. 5,104,310, European Patent Application No. 0131624B1, European Patent Application No. 120516, European Patent Application No. 159418B1, European Patent Application No. 176112, U.S. Patent No. 5,149,645, U.S. Patent No. 5,469,976, U.S. Patent No. 5,464,763, U.S. Patent No. 4,940,838, U.S. Patent No. 4,693,976, European Patent Application No. 116718, European Patent Application No. 290799, European Patent Application No. 320500, European Patent Application No. 604662, European Patent Application No. 627752, European Patent Application No. 0267159, European Patent Application No. 0292435, U.S. Patent No. 5,231,019, U.S. Patent No. 5,463,174, U.S. Patent No. 4,762,785, U.S. Patent No. 5,004,863, and U.S. Patent No. 5,159,135. The use of T-DNA-containing vectors for the transformation of plant cells has been intensively researched and sufficiently described in European Patent Application 120516; An *et al.,* (1985, EMBO J. 4:277–284), Fraley *et al.,* (1986, Crit. Rev. Plant Sci. 4:1-46), and Lee and Gelvin (2008, Plant Physiol. 146: 325-332), and is well established in the field.

The biology of T-DNA transfer from *Agrobacterium* to plant cells is known. See, *e.g.,* Gelvin (2003) Microbiol. Molec. Biol. Rev. 67:16-37; and Gelvin (2009) Plant Physiol. 150:1665–1676.. At minimum, at least a T-DNA right border repeat, but often both the right border repeat and the left border repeat of the Ti or Ri plasmid will be joined as the flanking region of the genes desired to be inserted into the plant cell. The left and right T-DNA border repeats are crucial *cis*-acting sequences required for T-DNA transfer. Various *trans*-acting components are encoded within the total *Agrobacterium* genome. Primary amongst these are the proteins encoded by the *vir* genes, which are normally found

as a series of operons on the Ti or Ri plasmids. Various Ti and Ri plasmids differ somewhat in the complement of vir genes, with, for example, *virF* not always being present. Proteins encoded by *vir* genes perform many different functions, including recognition and signaling of plant cell/bacteria interaction, induction of *vir* gene transcription, formation of a Type IV secretion channel, recognition of T-DNA border repeats, formation of T-strands, transfer of T-strands to the plant cell, import of the T-strands into the plant cell nucleus, and integration of T-strands into the plant nuclear chromosome, to name but a few. See, *e.g.,* Tzfira and Citovsky (2006) Curr. Opin. Biotechnol. 17:147-154.

If *Agrobacterium* strains are used for transformation, the DNA to be inserted into the plant cell can be cloned into special plasmids, for example, either into an intermediate (shuttle) vector or into a binary vector. Intermediate vectors are not capable of independent replication in *Agrobacterium* cells, but can be manipulated and replicated in common *Escherichia coli* molecular cloning strains. Such intermediate vectors comprise sequences are commonly framed by the right and left T-DNA border repeat regions, that may include a selectable marker gene functional for the selection of transformed plant cells, a cloning linker, a cloning polylinker, or other sequence which can function as an introduction site for genes destined for plant cell transformation. Cloning and manipulation of genes desired to be transferred to plants can thus be easily performed by standard methodologies in *E. coli*, using the shuttle vector as a cloning vector. The finally manipulated shuttle vector can subsequently be introduced into *Agrobacterium* plant transformation strains for further work. The intermediate shuttle vector can be transferred into *Agrobacterium* by means of a helper plasmid (*via* bacterial conjugation), by electroporation, by chemically mediated direct DNA transformation, or by other known methodologies. Shuttle vectors can be integrated into the Ti or Ri plasmid or derivatives thereof by homologous recombination owing to sequences that are homologous between the Ti or Ri plasmid, or derivatives thereof, and the intermediate plasmid. This homologous recombination (*i.e.* plasmid integration) event thereby provides a means of stably maintaining the altered shuttle vector in *Agrobacterium,* with an origin of replication and other plasmid maintenance functions provided by the Ti or Ri plasmid portion of the co-integrant plasmid. The Ti or Ri plasmid also comprises the vir regions comprising *vir* genes necessary for the transfer of the T-

DNA. The plasmid carrying the vir region is commonly a mutated Ti or Ri plasmid (helper plasmid) from which the T-DNA region, including the right and left T-DNA border repeats, have been deleted. Such pTi-derived plasmids, having functional *vir* genes and lacking all or substantially all of the T-region and associated elements are descriptively referred to herein as helper plasmids.

The superbinary system is a specialized example of the shuttle vector/homologous recombination system (reviewed by Komari *et al.*, (2006) In: Methods in Molecular Biology (K. Wang, ed.) No. 343: *Agrobacterium* Protocols (2nd Edition, Vol. 1) HUMANA PRESS Inc., Totowa, NJ, pp.15-41; and Komori *et al.,* (2007) Plant Physiol. 145:1155-1160). The *Agrobacterium tumefaciens* host strain employed with the superbinary system is LBA4404(pSB1). Strain LBA4404(pSB1) harbors two independently-replicating plasmids, pAL4404 and pSB1. pAL4404 is a Ti-plasmid-derived helper plasmid which contains an intact set of *vir* genes (from Ti plasmid pTiACH5), but which has no T-DNA region (and thus no T-DNA left and right border repeat sequences). Plasmid pSB1 supplies an additional partial set of *vir* genes derived from pTiBo542; this partial *vir* gene set includes the *virB* operon and the *virC* operon, as well as genes *virG* and *virD1*. One example of a shuttle vector used in the superbinary system is pSB11, which contains a cloning polylinker that serves as an introduction site for genes destined for plant cell transformation, flanked by right and left T-DNA border repeat regions. Shuttle vector pSB11 is not capable of independent replication in *Agrobacterium*, but is stably maintained as a co-integrant plasmid when integrated into pSB1 by means of homologous recombination between common sequences present on pSB1 and pSB11. Thus, the fully modified T-DNA region introduced into LBA4404(pSB1) on a modified pSB11 vector is productively acted upon and transferred into plant cells by Vir proteins derived from two different *Agrobacterium* Ti plasmid sources (pTiACH5 and pTiBo542). The superbinary system has proven to be particularly useful in transformation of monocot plant species. See Hiei *et al.*, (1994) Plant J. (6:271–282 and Ishida *et al.*, (1996) Nat. Biotechnol. 14:745–750.

In addition to the *vir* genes harbored by *Agrobacterium* Ti plasmids, other, chromosomally-borne virulence controlling genes (termed *chv* genes) are known to control certain aspects of the interactions of *Agrobacterium* cells and plant cells, and thus affect the

overall plant transformation frequency (Pan *et al.*, (1995) Molec. Microbiol. 17:259-269). Several of the chromosomally-borne genes required for virulence and attachment are grouped together in a chromosomal locus spanning 29 kilobases (Matthysse *et al.*, (2000) Biochim. Biophys. Acta 1490:208-212).

Regardless of the particular plasmid system employed, the *Agrobacterium* cells so transformed are used for the transformation of plant cells. Plant explants (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) can advantageously be cultivated with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants may then be regenerated from the infected plant material following placement in suitable growth conditions and culture medium, which may contain antibiotics or herbicides for selection of the transformed plant cells. The plants so obtained can then be tested for the presence of the inserted DNA.

These techniques for introducing foreign genetic material into plants can be used to introduce beneficial traits into the plants. For example, billions of dollars are spent each year to control insect pests and additional billions are lost to the damage they inflict. Synthetic organic chemical insecticides have been the primary tools used to control insect pests but biological insecticides, such as the insecticidal proteins derived from *Bacillus thuringiensis* (*Bt*), have played an important role in some areas. The ability to produce insect-resistant plants through the introduction of *Bt* insecticidal protein genes has revolutionized modern agriculture and heightened the importance and value of insecticidal proteins and their genes.

Several *Bt* proteins have been used to create the insect-resistant transgenic plants that have been successfully developed and in many cases registered and commercialized. These include Cry1Ab, Cry1Ca, Cry1Fa, and Cry3Bb in corn, Cry1Ac and Cry2Ab in cotton, and Cry3A in potato.

The commercial products expressing *Bt* proteins express a single protein except in cases where the combined insecticidal spectrum of 2 proteins is desired (*e.g.*, Cry1Ab and Cry3Bb in corn combined to provide resistance to lepidopteran pests and rootworm, respectively) or where the independent action of the proteins makes them useful as a tool

for delaying the development of resistance in susceptible insect populations (*e.g.*, Cry1Ac and Cry2Ab in cotton combined to provide resistance management for tobacco budworm).

That is, some of the qualities of insect-resistant transgenic plants that have led to rapid and widespread adoption of this technology also give rise to the concern that pest populations will develop resistance to the insecticidal proteins produced by these plants. Several strategies have been suggested for preserving the utility of *Bt*-based insect resistance traits which include deploying proteins at a high dose in combination with a refuge, and alternation with, or co-deployment of, different toxins (McGaughey *et al.* 1998, Nature Biotechnol.16:144-146).

If *Bt* proteins are selected for use in combination, they need to exert their insecticidal effect independently so that resistance developed to one protein does not confer resistance to the second protein (*i.e.*, there is not cross resistance to the proteins). A robust assessment of cross-resistance is typically made using populations of a pest species normally sensitive to the insecticidal protein that has been selected for resistance to the insecticidal proteins. If, for example, a pest population selected for resistance to "Protein A" is sensitive to "Protein B", we would conclude that there is not cross resistance and that a combination of Protein A and Protein B would be effective in delaying resistance to Protein A alone.

In the absence of resistant insect populations, assessments can be made based on other characteristics presumed to be related to mechanism of action and cross-resistance potential. The utility of receptor-mediated binding in identifying insecticidal proteins likely to not exhibit cross resistance has been suggested (U.S. Patent No. 6,855,873). The key predictor of lack of cross resistance integral to this approach is that the insecticidal proteins do not compete for receptors in a sensitive insect species.

In the event that two *Bt Cry* toxins compete for the same receptor, then if that receptor mutates in that insect so that one of the toxins no longer binds to that receptor and thus is no longer insecticidal against the insect, it might also be the case that the insect will also be resistant to the second toxin (which competitively bound to the same receptor). However, if two toxins bind to two different receptors, this could be an indication that the insect would not be simultaneously resistant to those two toxins.

Cry1Fa is useful in controlling many lepidopteran pests species including the European corn borer (ECB; *Ostrinia nubilalis* (Hübner)) and the fall armyworm (FAW; *Spodoptera frugiperda*), and is active against the sugarcane borer (SCB; *Diatraea saccharalis)*.

The Cry1Fa protein, as produced in corn plants containing event TC1507, is responsible for an industry-leading insect resistance trait for FAW control. Cry1Fa is further deployed in the HERCULEX®, SMARTSTAX™, and WIDESTRIKE™ products.

The ability to conduct (competitive or homologous) receptor binding studies using Cry1Fa protein is limited because the most common technique available for labeling proteins for detection in receptor binding assays inactivates the insecticidal activity of the Cry1Fa protein.

Cry1Ab and Cry1Fa are insecticidal proteins currently used (separately) in transgenic corn to protect plants from a variety of insect pests. A key pest of corn that these proteins provide protection from is the European corn borer (ECB). U.S. Patent Application No. 2008/0311096 relates in part to the use of Cry1Ab to control a Cry1F-resistant ECB population.

This application describes strains of *Agrobacterium tumefaciens* that have been modified to increase plant transformation frequency. The use of these strains provides novel plant transformation systems for the introduction of plant-expressible foreign genes into plant cells. In addition, these strains provide additional improvements which allow for increased transformation efficiency and provide significant advantages in overcoming operational disadvantage when transforming plants with foreign genes.

SUMMARY OF THE INVENTION

Agrobacterium strains that harbor transformation-enhancing genes on a plasmid capable of replication independently of the *Agrobacterium* chromosome, the Ti plasmid, and plant transformation binary vectors and methods for their use are described herein. The *Agrobacterium* strains are deficient in DNA recombination functions that result in instability or rearrangement of plant transformation binary vectors, and harbor transformation-enhancing genes on a plasmid capable of replication independently of the

Agrobacterium chromosome, the Ti plasmid, and plant transformation binary vectors. Additional *Agrobacterium* strains that harbor transformation-enhancing genes integrated into the *Agrobacterium* chromosome at a locus that does not interfere with or otherwise compromise the normal growth and plant transformation ability of the *Agrobacterium* cells and their use are also described.

In one embodiment of the methods described herein, a plant is transformed by contacting a cell of the plant with an *Agrobacterium* strain having at least one pTi helper plasmid comprising a 14.8 *KpnI* fragment of pSB1 and a pTi plasmid having at least one disarmed T-DNA region, the T-DNA region comprising at least a right T-DNA border and exogenous DNA adjacent to the border, wherein the plasmids have differing origins of replication relative to each other.

In a further embodiment of the methods described herein, a plant is transformed by contacting a cell of the plant with a bacterium of the genus Agrobacterium having a 14.8 KpnI VirBCDG fragment of pSB1 and a pTi plasmid having at least one disarmed T-DNA region, wherein the 14.8 KpnI VirBCDG fragment has been integrated into a neutral integration site of a chromosome of the bacterium.

In an additional embodiment of the methods described herein, an *Agrobacterium* strain includes at least one pTi helper plasmid comprising a 14.8 *KpnI* fragment of pSB1 and a pTi plasmid having at least one disarmed T-DNA region, wherein the plasmids have differing origins of replication relative to each other.

In a further embodiment, an *Agrobacterium* strain with transformation-enhancing properties includes a 14.8 *KpnI* VirBCDG fragment isolated from pSB1 and a pTi plasmid having at least one disarmed T-DNA region.

In another embodiment, a *nilA* genomic locus of *Agrobacterium tumefaciens* includes a polynucleotide sequence that is integrated into the *nilA* genomic locus.

In an additional embodiment, an *Agrobacterium* strain with a 14.8 *KpnI* VirBCDG fragment of SB1 is integrated into a neutral integration site on the *Agrobacterium* chromosome.

In another embodiment an *Agrobacterium* strain LB4404 includes a 14.8 *KpnI* VirBCDG fragment of pSB1on a pTi helper plasmid and a pTi plasmid having at least one disarmed T-DNA region and has exogenous DNA adjacent to at least one *Agrobacterium*

T-DNA border, wherein the plasmids have differing origins of replication relative to each other.

In a further embodiment an *Agrobacterium* strain LBA4404 includes at least one vir gene from a 14.8 *KpnI* VirBCDG fragment isolated from pSB1 integrated into a neutral integration site on the *Agrobacterium* chromosome.

In additional embodiments plants are provided that are made according to the transformation methods described herein.

In yet another embodiment, a fertile transgenic corn plant, or progeny thereof, expresses insecticidal amounts of Cry1Ca protein, Cry1F insecticidal protein, Cry1Ab1 insecticidal protein, and herbicide-tolerant amounts of AAD-1 protein, wherein the Cry1Ca, Cry1F, Cry1Ab1, and AAD1 proteins are collectively expressed from a single locus of recombinant DNA stably incorporated in the genome of the plant.

DESCRIPTION OF THE DRAWINGS

Figure 1. Shows a cloning scheme for the construction of plasmid pDAB9292.

Figure 2. Shows a map of plasmid pDOW3719.

Figure 3. Shows a cloning scheme for the construction of plasmid pDAB9698.

Figure 4. Shows maps of binary vector plasmids pDAB101513 and pDAB101514.

Figure 5. Shows a map of binary vector plasmid pDAB101556.

DETAILED DESCRIPTION OF THE INVENTION

Strains of *Agrobacterium* differ from one another in their ability to transform plant cells. Wild-type, oncogenic *Agrobacterium* strains are known for their ability to induce crown galls (tumorous overgrowths) on many host plants, especially dicot species. This transformation of normally growing plant cells into non-self regulated tumor cells comes about as the result of the transfer of specialized DNA sequences (T-DNA), which encodes plant expressible genes encoding plant hormones, from the tumor-inducing (Ti) plasmid into the plant cells, wherein they are stably integrated into plant chromosomes. The Ti plasmid from strain Bo542 (*i.e.* pTiBo542) is notable in that, when placed in some *Agrobacterium* chromosomal backgrounds, it promotes the induction of especially large, vigorously-growing tumors on some plants (Hood *et al.*, (1986) J. Bacteriol. 168:1291–

1301). The genes responsible for this "supervirulence" phenotype reside on pTiBo542 outside the T-DNA regions. Further work found that a plasmid containing a "15.8" kilobase pair (kbp) *KpnI* fragment derived from pTiBo542 and which contained the entire *virG, virB,* and *virC* operons promoted increased tumor formation by strain A281, when compared to strains lacking the plasmid (Jin *et al.,* (1987) J. Bacteriol. 169:4417-4425). The *virG* gene of pTiBo542 is believed to be responsible for the supervirulent phenotype of *Agrobacterium strain* A281. *virG* from pTiBo542 causes a 1.7-fold increase in *virB* expression compared with *virG* from pTiA6, due to differences between the two genes in the promoter regions, coding sequences, and 3' untranslated regions (Chen *et al.*, (1991) Molec. Gen. Genet. 230:302-309). Thus, the *virG* gene from pTiBo542 can be advantageously used to promote higher T-DNA transfer efficiencies, and thus higher plant transformation frequencies, especially when present on a large *KpnI* fragment of the pTiBo542 plasmid that also harbors the pTiBo542 *virB* and *virC* operons.

The complete, annotated sequence of pTiBo542 was submitted to GENBANK as Accession Number DQ058764 on May 12, 2005. Examination of the *KpnI* restriction fragment map and gene annotations reveals that the entire *virB* operon (which includes the genes *virB1*, *virB2*, *virB3*, *virB4*, *virB5*, *virB6*, *virB7*, *virB8*, *virB9*, *virB10*, and *virB11*), the *virG* gene, the *virC* operon (which comprises genes *virC1* and *virC2*) and the part of the *virD* operon comprising gene *virD1* are isolatable on a *KpnI* fragment comprising 14,815 base pairs (bp). Assumedly, the size of the "15.8 kbp" *KpnI* fragment referred to in Jin *et al.*, (*supra.*) was estimated from agarose gel mobility of the fragment, and that the true size of the referenced fragment is, in fact, 14.8 kbp. One skilled in the field of molecular biology will understand that size estimation of such large DNA fragments by means of agarose gel electrophoresis mobility can differ from the true fragment size determined by DNA sequence analysis by 1 kbp or more. For ease of description, this fragment derived from pTiBo542 will be referred to herein as the 14.8 *KpnI* VirBCDG fragment.

An embodiment of methods described herein includes uses of the transformation-enhancing properties encoded on the 14.8 *KpnI* VirBCDG fragment isolated from pSB1 in *Agrobacterium* strains harboring at least one disarmed pTi helper plasmid, wherein the 14.8 *KpnI* VirBCDG fragment is borne on a plasmid having a replication origin of an incompatibility group other than IncP to transform a plant. A further embodiment includes

the *Agrobacterium* strain as described for use in the method. A T-DNA region to be introduced to a plant using this *Agrobacterium* strain can be borne on a plasmid having a T-DNA region adjacent to at least one *Agrobacterium* T-DNA border, the plasmid having a replication origin of an IncP incompatibility group or an incompatability group that is compatible with the incompatibility group of the 14.8 *KpnI* VirBCDG fragment that is borne on a plasmid having a replication origin of an incompatibility group other than IncP. The T-DNA region of this plasmid can be adjacent right and left *Agrobacterium* T-DNA borders.

Plasmids are assigned to incompatibility groups (genotypic designation: *inc*; group designation: Inc) based on sequences contained in the plasmid. The *inc* determinant typically serves to prevent other plasmids of the same or related incompatibility group from coexisting in the same host, and helps maintain a certain copy number of the plasmid within the cell. See, *e.g.*, Fernandez-Lopez, *et al.*, (2006) FEMS Microbiol. Rev. 30:942-66; and Adamczyk and Jagura-Burdzy (2003) Acta Biochim. Pol. 50:425-53. Two plasmids are incompatible if either is less stable in the presence of the other than it was by itself. Competition for cell resources can result when two plasmids of the same incompatibility group are found in the same cell. Whichever plasmid is able to replicate faster, or provides some other advantage, will be represented to a disproportionate degree among the copies allowed by the incompatibility system. Surprisingly, plasmids can also be incompatible when they both possess the same functions for partitioning themselves into daughter cells.

Plasmids typically fall into only one of the many existing incompatibility groups. There are more than 30 known incompatibility groups. Plasmids belonging to incompatibility group IncP have been studied thoroughly and a large number of plasmids which derive from this IncP group have been constructed (Schmidhauser *et al.*, (1988) Biotechnology 10:287-332). Exemplary plasmids containing the IncP incompatibility group include: pMP90RK, pRK2013, pRK290, pRK404, and pRK415. These plasmids may be maintained in numerous bacterial species including *E. coli* and *Agrobacterium tumefaciens*. Examples of other incompatibility groups include, but are not limited to; IncN, IncW, IncL/M, IncT, IncU, IncW, IncY, IncB/O, IncFII, IncII, IncK, IncCom9, IncFI, IncFII, IncFIII, IncHIl, IncHI2, IncX, IncA/C, IncD, IncFIV, IncFV/FO, IncFVI, IncHl 3, IncHII, Inc12, IncI, IncJ, IncV, IncQ, and the like, including variants thereof, *e.g.*,

exhibiting substantial sequence or functional relationship. Table 1 lists several commonly known incompatibility groups and provides examples of plasmids which represent these incompatibility groups (this listing of incompatability groups and plasmids is provided by way of example only and is not intended to be limiting on the incompatibility groups and plasmids useful with the *Agrobacterium* strains and methods described herein).

Another embodiment of the methods described herein includes uses of transformation-enhancing properties encoded on the 14.8 *KpnI* VirBCDG fragment isolated from pSB1 in *Agrobacterium* strains having a deficiency in RecA function, and harboring at least one disarmed pTi helper plasmid, wherein the 14.8 *KpnI* VirBCDG fragment is borne on a plasmid having a replication origin of an incompatibility group other than IncP. A further embodiment includes the *Agrobacterium* strain as described for use in the method. A T-DNA region to be introduced to a plant using this *Agrobacterium* strain can be borne on a plasmid having a T-DNA region adjacent to at least one *Agrobacterium* T-DNA border, the plasmid having a replication origin of an IncP incompatibility group or an incompatability group that is compatible with the incompatibility group of the 14.8 *KpnI* VirBCDG fragment that is borne on a plasmid having a replication origin of an incompatibility group other than IncP.

Yet another embodiment of the methods described herein includes uses of the transformation-enhancing properties encoded on the 14.8 *KpnI* VirBCDG fragment isolated from pSB1, and harboring at least one disarmed pTi helper plasmid, wherein the 14.8 *KpnI* VirBCDG fragment is integrated into a chromosomally located neutral integration site of an *Agrobacterium* strain different from strain C58. A further embodiment includes the *Agrobacterium* strain as described for use in the method. A T-DNA region to be introduced to a plant using this *Agrobacterium* strain further comprises a plasmid having a T-DNA region adjacent to at least one *Agrobacterium* T-DNA border.

Although superbinary systems are known, for example, see WO 94/00977A1, WO 95/06722A1, and WO 95/16031A1, and are further described by Komari *et al.*, (*supra*), and Komori *et al.,* (*supra*), these systems possess a number of disadvantages. An operational disadvantage of the superbinary system, which is overcome by the *Agrobacterium* strains and methods described herein, is the necessity for formation of a co-integrant plasmid between pSB1 and pSB11 (and its derivatives) as the means by which the altered T-DNA

borne on pSB11 derivatives is to be stably maintained in *Agrobacterium*. This co-integration event generates a pair of large (*ca.* 2.3 kbp) directly repeated sequences due to recombination between the homologous regions of pSB1 and pSB11. As is well known to those skilled in the field of molecular biology, large repeated sequences such as these are preferred targets for intramolecular recombination that leads eventually to DNA deletions and other rearrangements, particularly when the repeats are a part of plasmid structure. In the *Agrobacterium* superbinary system, such rearrangements may lead to partial rearrangement or complete loss of the T-DNA region introduced by pSB11 derivatives, ultimately resulting in little or no transfer of intact desired foreign genes into the host plant cells.

A further disadvantage to the above-described superbinary system, and which is also overcome by the *Agrobacterium* strains and methods described herein, is that the formation of the co-integrant plasmid between pSB1 and pSB11 derivatives generates a large plasmid (minimally, greater than 43 kbp) having two distinct ColE1-type (incompatibility group pMB1/ColE1) origins of replication (*ori*), as well as a third *ori* derived from the RK2 plasmid (incompatibility group IncP). Although in normal circumstances the ColE1 *ori* is nonfunctional in *Agrobacterium,* genomic mutations are known which allow the stable maintenance of plasmids having a ColE1 *ori* in *Agrobacterium* (Ruslyakova *et al.*, (1999) Russian J. Genet. 35:327-331). In cells having such mutations, a plasmid such as the pSB1::pSB11 derivative co-integrant having 3 functional origins of replication would be expected to be highly unstable. Thus, the superbinary system has imperfections that are advantageously addressed by elements of the *Agrobacterium* strains and methods for transforming plants described herein.

The DNA structure of the foreign gene or genes destined for introduction and expression in transgenic plant cells by *Agrobacterium*-mediated transformation can have a profound influence on the stability of the binary vector plasmid or shuttle vector plasmid harboring those genes in cells of *Escherichia coli* and *Agrobacterium*. Instability is particularly manifested when the foreign genes comprise gene components that are employed multiple times within the gene constructs. For example, it is not uncommon that a particular plant-expressible promoter may be used to drive the expression of different protein coding regions in a transgenic plant. Other gene components such as 3' untranslated

regions (3'UTR) (*i.e.* transcription termination and polyadenylation addition determining sequences) and even highly similar protein coding regions may be duplicated or present in multiple copies within a single T-DNA region. As mentioned above, these repeated sequence elements, which may exist in either inverted or directly repeated orientations, are targets for intramolecular recombinations that may lead to DNA deletions and other rearrangements, particularly as the repeats are a part of plasmid structure.

Multiple specialized strains of *E. coli* have been developed to serve as molecular cloning hosts that help to overcome such instability difficulties (*e.g.* STBL2™, STBL3™, and STBL4™ strains offered by INVITROGEN; Carlsbad, CA). A feature common to all such *E. coli* cloning strains is the presence of a genomic mutation in a *recA* gene. The RecA protein is a multifunctional enzyme that plays a role in homologous recombination, DNA repair, and induction of the bacterial SOS response. In the homologous recombination process, the protein functions as a DNA-dependent ATPase, promoting synapsis, heteroduplex formation and strand exchange between homologous DNAs. Thus, cells deficient in RecA function are more prone to tolerate homologous DNA sequences without rearrangement or deletion.

RecA deficient strains of *Agrobacterium* have been developed to help address the instability problems observed when cloning large DNA fragments containing repeated sequences (Klapwicj *et al.*, (1979) Molec. Gen. Genet. 173:171-175; Farrand *et al.*, (1989) J. Bacteriol. 171:5314-5321; Lazo *et al.*, (1991) Bio/Technology 9:963-967). These strains have proven useful in helping stabilize high molecular weight transforming constructs in some cases (Frary and Hamilton, (2001) Transgenic Res. 10:121-132), but not in all instances (Song *et al.*, (2003) Theor. Appl. Genet.107:958-964). Thus, *Agrobacterium* chromosomal backgrounds that are *recA* defective in developing strains that are highly efficient in plasmid maintenance and plant transformation capability can be advantageously used. In addition to using *Agrobacterium* chromosomal backgrounds that are *recA* defective in developing strains for use in the methods described herein, the *recA* functionality can be deactivated in an existing or produced strain to make that strain useful in the methods described herein. See, *e.g.,* Farrand *et al.* (*supra*). For example, a strain can be developed with RecA functionality and any chromosomal additions desired, *e.g.*, the addition of *vir* genes, can be made then the RecA functionality disabled.

BIBAC vectors designed to enable efficient transformation of large DNA fragments into plant and non-plant host cells can be used. See, *e.g.*, U.S. Patent No. 5,733,744, U.S. Patent No. 5,977,439, and U.S. Patent Application No. 2002/0123100A1. One *Agrobacterium* strain that can be utilized with the BIBAC vectors is the RecA-deficient strain UIA143 developed by Farrand *et al.,* (*supra*). Refinements to the BIBAC system have used subsets of the genes harbored on the 14.8 *KpnI* VirBCDG fragment in combination with other *vir* genes to enhance the plant transformation capability of engineered *Agrobacterium* strains. In particular, the *virG* gene from the 14.8 *KpnI* VirBCDG fragment has been employed alone or in combination with the *virE1* and *virE2* genes from pTiA6 in the UIA143 RecA-deficient strain. See, *e.g.,* Hamilton *et al.*, (1996) Proc. Natl. Acad. Sci. 93:9975-9979; Hamilton, (1997) Gene 200:107-116; Frary and Hamilton, (*supra*).

In addition, a suitable vector used to transform plant cell using the methods described herein can contain a selectable marker gene encoding a protein that confers on the transformed plant cells resistance to an antibiotic or a herbicide. The individually employed selectable marker gene may accordingly permit the selection of transformed cells while the growth of cells that do not contain the inserted DNA can be suppressed by the selective compound. The particular selectable marker gene(s) used may depend on experimental design or preference, but any of the following selectable markers may be used, as well as any other gene not listed herein that could function as a selectable marker. Examples of selectable markers include, but are not limited to, genes that provide resistance or tolerance to antibiotics such as Kanamycin, G418, Hygromycin, Bleomycin, and Methotrexate, or to herbicides, such as Phosphinothricin (Bialaphos), Glyphosate, Imidazolinones, Sulfonylureas, Triazolopyrimidines, Chlorosulfuron, Bromoxynil, and DALAPON.

In addition to a selectable marker, a reporter gene may also be used. In some instances a reporter gene could be used without a selectable marker. Reporter genes are genes that typically do not provide a growth advantage to the recipient organism or tissue. Reporter genes typically encode for a protein that provides for a phenotypic change or enzymatic property. Suitable reporter genes include, but are not limited to, those that

encode glucuronidase (GUS), firefly luciferase, or fluorescent proteins such as green fluorescent protein and yellow fluorescent protein.

In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue may include, but is not limited to, embryogenic tissue, callus tissue types I and II, hypocotyl, and meristem. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques within the skill of the art. One skilled in the field of plant transformation will understand that multiple methodologies are available for the production of transformed plants, and that they may be modified and specialized to accommodate biological differences between various host plant species.

Regardless of the particular transformation technique employed, the foreign gene can be incorporated into a gene transfer vector adapted to express the foreign gene in a plant cell by including in the vector a plant promoter. In addition to plant promoters, promoters from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoters of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the 35S and 19S promoters of cauliflower mosaic virus (CaMV), a promoter from sugarcane bacilliform virus, and the like may be used. Plant-derived promoters include, but are not limited to, ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu) promoter, beta-conglycinin promoter, phaseolin promoter, ADH (alcohol dehydrogenase) promoter, heat-shock promoters, ADF (actin depolymerization factor) promoter, and tissue specific promoters. Promoters may also contain certain enhancer sequence elements that may improve the transcription efficiency. Typical enhancers include, but are not limited to, alcohol dehydrogenase 1 (ADH1) intron 1 and ADH1-intron 6. Constitutive promoters may be used. Constitutive promoters direct continuous gene expression in nearly all cells types and at nearly all times (*e.g.*, actin promoter, ubiquitin promoter, CaMV 35S promoter). Tissue specific promoters are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds. Examples of other promoters that may be used include those that are active during a certain stage of the plant's development, as well as active in specific plant tissues and organs. Examples of such promoters include, but are not limited to, promoters that are root specific, pollen-specific,

embryo specific, corn silk specific, cotton fiber specific, seed endosperm specific, and phloem specific.

Under certain circumstances, the use of an inducible promoter may be desirable. An inducible promoter is responsible for expression of genes in response to a specific signal, such as physical stimulus (*e.g.* heat shock gene promoters); light (*e.g.* Ribulose-bis-phosphate 1,5 carboxylase promoter); hormone (*e.g.* glucocorticoid); antibiotic (*e.g.* Tetracycline); metabolites; and stress (*e.g.* drought). Other desirable transcription and translation elements that function in plants also may be used, such as, for example, 5' untranslated leader sequences, RNA transcription termination sequences and poly-adenylate addition signal sequences. Any suitable plant-specific gene transfer vector known to the art may be used.

Transgenic crops containing insect resistance (IR) traits are prevalent in corn and cotton plants throughout North America, and usage of these traits is expanding globally. Commercial transgenic crops combining IR and herbicide tolerance (HT) traits have been developed by multiple seed companies. These include combinations of IR traits conferred by *Bt* (*Bacillus thuringiensis*) insecticidal proteins and HT traits such as tolerance to Acetolactate Synthase (ALS) inhibitors such as Sulfonylureas, Imidazolinones, Triazolopyrimidine, Sulfonanilides, and the like, Glutamine Synthetase (GS) inhibitors such as Bialaphos, Glufosinate, and the like, 4-HydroxyPhenylPyruvate Dioxygenase (HPPD) inhibitors such as Mesotrione, Isoxaflutole, and the like, 5-EnolPyruvylShikimate-3-Phosphate Synthase (EPSPS) inhibitors such as Glyphosate and the like, and Acetyl-Coenzyme A Carboxylase (ACCase) inhibitors such as Haloxyfop, Quizalofop, Diclofop, and the like. Other examples are known in which transgenically provided proteins provide plant tolerance to herbicide chemical classes such as phenoxy acids herbicides and pyridyloxyacetates auxin herbicides (see WO 2007/053482A2), or phenoxy acids herbicides and aryloxyphenoxypropionates herbicides (see WO 2005107437A2,A3). The ability to control multiple pest problems through IR traits is a valuable commercial product concept, and the convenience of this product concept is enhanced if insect control traits and weed control traits are combined in the same plant. Further, improved value may be obtained via single plant combinations of IR traits conferred by a *Bt* insecticidal protein with one or more additional HT traits such as those mentioned above, plus one or more additional input

traits (*e.g.* other insect resistance conferred by *Bt*-derived or other insecticidal proteins, insect resistance conferred by mechanisms such as RNAi and the like, disease resistance, stress tolerance, improved nitrogen utilization, and the like), or output traits (*e.g.* high oils content, healthy oil composition, nutritional improvement, and the like). Such combinations may be obtained either through conventional breeding (*e.g.* breeding stack) or jointly as a novel transformation event involving the simultaneous introduction of multiple genes (*e.g.* molecular stack). Benefits include the ability to manage insect pests and improved weed control in a crop plant that provides secondary benefits to the producer and/or the consumer. Thus, the *Agrobacterium* strains and methods described herein can be used to provide transformed plants with combinations of traits that comprise a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic issues.

The *virG* genes of various pTi plasmids have been studied to understand their ability to enhance plant transformation frequency. Liu *et al.*, (1992, Plant Molec. Biol. 20:1071-1087) found that extra copies of *virG* genes from multiple sources (*i.e.* from different pTi plasmids, but including pTiBo542) enhanced the transient transformation of some plants, and the magnitude of the effect depended on the identity of the helper pTi plasmid with which the particular *virG* gene was paired. A mutant of a *virG* gene (presumably from pTiA6), named *virGN54D* (the mutation replaces amino acid Asn54 with Asp), is constitutively expressed in *Agrobacterium* (induction of wild-type *virG* genes requires an acidic pH, a high monosaccharide concentration, and the presence of phenolic inducers, such as acetosyringone). See Pazour *et al.*, (1992) J. Bacteriol. 174:4169-4174. V*irGN54D* of pTiA6 was effective in enhancing maize transformation, whereas multiple copies of the parent wild-type *virG* were ineffective. See Hansen *et al.*, (1994) J. Bacteriol. 174:4169-4174. A "ternary" (*i.e.* three-plasmid) system wherein a copy of the constitutive mutant *virGN54D* gene from pTi15955 was co-resident on a pBBR1-derived plasmid in *Agrobacterium tumefaciens* strain LBA4404 that contained the disarmed pTi helper plasmid pAL4404 and a binary vector harboring genes for plant transformation has been described. See van der Fits *et al.*, (2000) Plant Molec. Biol. 43:495-502. The constitutively expressed *virGN54D* gene was found to dramatically increase both transient and stable transformation efficiencies of several plant species. Plasmids containing the pBRR1 replication control

region cannot be classed as belonging to any known incompatibility group and, thus, may co-exist with a broad range of other plasmids in a single host. Further, the abilities of various combinations of *vir* genes to affect plant transformation efficiencies in tobacco, cotton and rice have been tested, specifically: the mutant v*irGN54D* gene derived from pTiA6, the *virG* gene from pTiBo542, the *VirE1/E2* genes from pTiA6, and a combination of the latter two gene sets. See Park *et al.*, (2000) Theor. Appl. Genet. 101:1015-1020. Increases in transformation efficiencies were observed with some plant species and additional copies of *vir* genes.

European Patent Application No. 2042602A1 and U.S. Patent Application No. 2010/0132068A1 describe cosmid binary vectors and "booster" plasmids that, when present in an *Agrobacterium* cell harboring a pTi helper plasmid, constitute further examples of ternary plasmid systems. Booster plasmids as disclosed therein possess a replication origin of the IncW incompatibility group, and comprise plasmid pVGW, having the *virGN54D* gene, and plasmid pVGW2, which is a derivative of pVGW having modifications to facilitate cloning and selection.

The functions encoded by chromosomal genes in *Agrobacterium* have classically been determined by two genetic approaches. The first, or forward genetics method, entails obtaining a molecular clone of the gene to be studied, followed by placement of the cloned gene in a genetic environment wherein a "gain of function" phenotype can be assessed. A second, or "reverse genetics method," requires disruption of the genes' structure by insertion or deletion of sequences in or around the gene in the chromosome, followed by determination of which proteins or phenotypes have been removed by the loss of gene function. This is the approach used to construct the previously described RecA deficient mutant of strain C58. See Farrand *et al., supra*. Those skilled in the field of genetic manipulation of *Agrobacterium* cells will understand that diverse vectors and numerous methods have been described to enable such gene disruption experiments. The method has proven to be particularly useful when used to identify genes that are not involved in vitality, growth, and plant transformation capability of the mutated strain. One such genetic locus in *Agrobacterium* strain C58 is the *pgl/picA* locus. See, Lee *et al.*, (2001) Plant Microbe Interact. 14:577-579; and Lee (2006) In: Methods in Molecular Biology (K. Wang, ed.) No. 343: *Agrobacterium* Protocols (2nd Edition, Vol. 1) HUMANA PRESS Inc., Totowa, NJ.

pp.55-66. Cells in which a *virD2* gene has been integrated into this chromosomal locus by homologous recombination were found to have a plant transformation phenotype identical to that resulting from *A. tumefaciens* strains harboring the *virD2* gene located on a replicating plasmid. See Lee *et al.*, *supra*. Further, a T-DNA region integrated into the *pgl/picA* locus of C58 may be functionally delivered to the plant cell (Oltmanns *et al.* 2010. Plant Physiol. 152:1158-1166). Thus, in strain C58, the *pgl/picA* locus can serve as a "neutral integration site" for introduction of genes into the C58 chromosome. As used herein, "neutral integration site" refers to a gene or chromosomal locus, natively present on the chromosome of an *Agrobacterium* cell, whose normal function is not required for the growth of the cell or for the capability of the cell to perform all the functions required for plant transformation. When disrupted by the integration of a DNA sequence not normally present within that gene, the cell harboring a disrupted neutral integration site gene can productively perform plant transformation. By way of example, Hoekema *et al.* (1984, EMBO J. 3:2485-2490) demonstrated that a functional T-region integrated into an uncharacterized locus in the C58 chromosome by means of Tn3 transposition was productively transferred to plant cells.

The *Agrobacterium* strains discussed herein can be used advantageously to introduce one or more genes into a plant, e.g., to provide individual or multiple insecticidal or herbicidal properties to the plant. For example, the *Agrobacterium* strains can be used to introduce one or more, two or more, three or more, four or more, five or more, or six or more genes into a plant. Using the *Agrobacterium* strains described herein, the polynucleotide containing the selectable gene sequences is inserted into a single location in the plant cell when the plant cell is transformed. In terms of the size of the T-DNA regions used to insert the genes, the T-DNA regions can be equal to or greater than 15,000 nucleotide base pairs, greater than or equal to 20,000 nucleotide base pairs, equal to or greater than 25,000 nucleotide base pairs, equal to or greater than 26,000 nucleotide base pairs, equal to or greater than 27,000 nucleotide base pairs, equal to or greater than 28,000 nucleotide base pairs, equal to or greater than 29,000 nucleotide base pairs, or equal to or greater than 30,000 nucleotide base pairs. When using the *Agrobacterium* strains described herein, the selectable gene sequences can have equal to or greater than 60%, equal to or greater than 65%, equal to or greater than 67%, equal to or greater than 69.5%, equal to or

greater than 70%, equal to or greater than 75%, or equal to or greater than 80% sequence homology and retain their transcribable sequence identities. The types of genes that can be introduced can encode insecticidal proteins, herbicidal proteins, or a mixture of insecticidal proteins and herbicidal proteins. Specific examples of genes that can be introduced include the genes encoding the Cry1Ca insecticidal protein, Cry1F insecticidal protein, Cry1Ab1 insecticidal protein, and AAD1 herbicidal protein, which can be introduced in various combinations or as a set including all four. Monocotyledonous (monocot) and dicotyledonous (dicot) species can be transformed using these *Agrobacterium* strains.

Also disclosed herein is the *nilA* genomic locus of *Agrobacterium tumefaciens*, into which a polynucleotide sequence can be integrated. Such an integrated polynucleotide sequence can include any *vir* gene or *vir* operon or other useful genes. Examples 17 - 20 show the identification, characterization, and use of the *nilA* genomic locus of *Agrobacterium tumefaciens* as well as the production of an *Agrobacterium tumefaciens* strain with multiple *vir* genes located on the chromosome. The *nilA* genomic locus, or any locus which shares 85-100% nucleotide sequence identity, could be identified in other *Agrobacterium* strains using the techniques for identification and characterization described herein, and any such identified *nilA* loci could be used in a manner similar to that described herein to integrate *vir* or other suitable genes which can, *e.g.*, increase the efficiency of plant transformation. The techniques for identification and characterization of such a genomic locus described herein could also be used to identify other neutral integration sites on the *Agrobacterium* chromosome at which polynucleotide sequences containing *vir* or other genes can be integrated such that the *Agrobacterium* strain remains capable of transforming plants. Some chromosomal sites are already known that could be used as neutral integration sites, for example, the RecA site in a RecA deficient strain, and the *pgl/picA* locus in *Agrobacterium tumefaciens* strain C58. However, there is a need to identify new neutral sites in *Agrobacterium tumefaciens* strains besides C58, as the *pgl/picA* locus is not detected in some other strains, for example, strain LBA4404 (Oltmanns *et al., supra*). Additional chromosomal sites which can be used as neutral integration sites are described in U.S. Pat. No. 6,323,396. Thus, an *Agrobacterium* strain with a *vir* gene integrated into a neutral integration site on the *Agrobacterium* chromosome is also

disclosed. Such an *Agrobacterium* strain could use a *nilA* genomic locus or other neutral integration site for the integration of *vir* genes.

Multiple types of useful genes could be added to the chromosome in this way making the use of T-helper plasmids unnecessary. For example, additional *vir* genes and multiple copies of useful vir genes from different strains could be used.

Also disclosed herein is an *Agrobacterium* strain containing *vir* genes on a helper plasmid having a replication origin of an incompatibility group other than IncP and a plasmid having a T-DNA region adjacent to at least one *Agrobacterium* T-DNA border, the plasmid having a replication origin of an IncP incompatibility group.

Further disclosed are plants made by the methods described herein using the *Agrobacterium* strains described herein. Such plants stably integrate any T-DNA regions introduced using the methods described herein. Further, such plants express any genes and exhibit any genetic traits conferred by those T-DNA regions. Additionally, any progeny of the plants made by the methods described herein using the *Agrobacterium* strains described herein stably produce any genes and exhibit any genetic traits conferred by those T-DNA regions found in the parent.

In a specific embodiment, a plant is described that stably expresses Cry1Ca insecticidal proteins, Cry1F insecticidal proteins, Cry1Ab1 insecticidal proteins, and AAD1 herbicidal proteins. This plant, for example, can be maize.

While certain example *Agrobacterium* strains are described herein, the functionality discussed could be moved to other *Agrobacterium* strains with the same criteria, *e.g.*, other strains which are deficient in RecA or could be made deficient in RecA. Examples of other strains that could be used with the strains and methods described herein include, but are not limited to, *Agrobacterium tumefaciens* strain C58, *Agrobacterium tumefaciens* strain Chry5, *Agrobacterium rhizogenes* strains, *Agrobacterium tumefaciens* strain EHA101, *Agrobacterium tumefaciens* strain EHA105, *Agrobacterium tumefaciens* strain MOG101, and *Agrobacterium tumefaciens* strain T37.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples that illustrate procedures for utilizing the *Agrobacterium* strains and practicing the methods described herein. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted. All temperatures are in degrees Celsius.

Unless specifically indicated or implied, the terms “a”, “an”, and “the” signify “at least one” as used herein.

EXAMPLE 1: Construction of a deletion variant of plasmid pUCD2

Construction of plasmid pUCD2 was described by Close *et al.*, (1984, Plasmid 12:111-118), and the complete 13,239 bp DNA sequence is disclosed for the first time herein as SEQ ID NO:1. pUCD2 harbors four genes conferring bacterial resistance to antibiotics: specifically, resistance to Spectinomycin, Kanamycin, Tetracycline, and Ampicillin (Figure 1). Standard molecular biology methods, as taught, for example, in Sambrook *et al.*, (1989, *Molecular Cloning: A Laboratory Manual* (2nd Edition., COLD SPRING HARBOR LABORATORY PRESS, Plainview, N.Y.) and Ausubel *et al.*, (1995, *Current Protocols in Molecular Biology,* (GREENE PUBLISHING AND WILEY-INTERSCIENCE, New York), and updates thereof, were employed in this and other steps described in this example and in other examples of this disclosure. A first modification to pUCD2 was made by cleaving pUCD2 DNA with restriction enzymes *Sac I* and *Sac II* and ligation to a mostly double-stranded oligonucleotide fragment having appropriate overhanging "sticky ends" compatible with *Sac I*- or *Sac II* generated overhangs. This double-stranded oligonucleotide (Figure1) was created by annealing two complementary oligonucleotide sequences, disclosed as SEQ ID NO:2 and SEQ ID NO:3. The sequences of the oligonucleotides of SEQ ID NO:2 and SEQ ID NO:3 are designed to restore a functional Kanamycin resistance gene upon ligation with pUCD2 DNA cleaved with *Sac I* and *Sac II*. This manipulation created plasmid pDAB9290 (Figure 1), which differs from pUCD2 by the deletion of the coding region for Spectinomycin resistance, elimination of a *Kpn I* restriction enzyme recognition site from within the coding region for Kanamycin resistance, and creation of a new *Kpn I* site downstream of the Kanamycin resistance coding region.

DNA of plasmid pDAB9290 was further manipulated to render inoperative the genes encoding Tetracycline resistance and Ampicillin resistance by first cleaving with restriction enzymes *Pst I* and *Sal I*, treating the overhanging ends left by these enzymes with the QUICK BLUNTING™ kit (NEW ENGLAND BIOLABS; Ipswich, MA) to create blunt ends, and self ligation to circularize the fragments thus produced. The resulting plasmid (pDAB9291) retains only the Kanamycin bacterial antibiotic resistance gene, and has a unique site for cleavage by *Kpn I* downstream of the Kanamycin resistance gene. The sequence of pDAB9291 is disclosed as SEQ ID NO:4. Plasmid pDAB9291 has two origins of replication, one (colE1 incompatibility group) derived from plasmid pBR322, and a second derived from plasmid pSa (incompatibility group W). Thus, plasmid pDAB9291 is capable of medium-copy-number maintenance in *E. coli* and *Agrobacterium*.

EXAMPLE 2: Cloning of a 14.8 *Kpn I* virBCDG fragment into pDAB9291

A 14.8 kbp *Kpn I* fragment containing the *virG*, *virB*, and *virC* operons and *virD1* from the "supervirulent" pTiBo542 (Figure 1) was isolated from plasmid pSB1 (Komari *et al.*, *supra*; and Komori *et al.*, *supra*), and cloned into the unique *Kpn I* site of pDAB9291. Plasmids containing each of the two possible orientations of the insert fragment were obtained, and were named pDAB9292 and pDAB9293. One plasmid, pDAB9292 (Figure 1) was selected for further work. The DNA sequence of pDAB9292 is disclosed as SEQ ID NO:5.

EXAMPLE 3: Construction of a RecA-deficient *Agrobacterium* strain harboring the helper plasmid pTiEHA105

Agrobacterium strain UIA143 is a RecA-deficient strain having the C58 genetic background and was constructed and described by Farrand *et al.*, (*supra*). The chromosomal *recA* gene was deleted and replaced with a gene cassette conferring resistance to Erythromycin at 150 µg/mL. The UIA143 strain contains no Ti plasmid or Ti plasmid derivative.

Agrobacterium strain EHA105, constructed and described by Hood *et al.*, (1993, Transgenic Research 2:208-221), harbors a helper plasmid (herein called pTiEHA105)

derived from the "supervirulent" pTiBo542 plasmid. Plasmid pTiEHA105 DNA was prepared from strain EHA105 and introduced by electroporation into cells of strain UIA143 made electrocompetent by standard methods (Weigel and Glazebrook, (2002) *Arabidopsis:* A Laboratory Manual. COLD SPRING HARBOR PRESS, Cold Spring Harbor, NY, 354 pages; Mersereau *et al.*, (1990) Gene 90:149-151; Mattanovich, *et al.,* (1989) Nucl. Acids Res. 17:6747)). Strain UIA143 cells transformed with pTiEHA105 were selected by their ability to grow on AB minimal medium (Watson, *et al.*, (1975) J. Bacteriol. 123:255-264) using purified agar and mannopine (2 mg/mL) as a sole source of carbon and nitrogen for growth (Guyon *et al.*, (1980) Proc. Natl. Acad. Sci. 77:2693-2697; Dessaux *et al.*, (1987) Molec. Gen. Genet. 208:301-308).

The presence of pTiEHA105 was verified by polymerase chain reaction (PCR) using primers designed to amplify fragments of the pTiBo542 *virD2* and *virG* genes, and further characterized by Southern blot analysis of total DNA prepared from candidate colonies probed with 32P-labeled DNA of pTiEHA101 purified by cesium chloride gradient centrifugation. This *Agrobacterium* strain (*i.e.* UIA143 containing pTiEHA105) is named DA2552.

EXAMPLE 4: Construction of a RecA-deficient *Agrobacterium* strain harboring the helper plasmid pTiC58Δ

Strain Z707 was derived by replacing the entire T-DNA region of the pTiC58 plasmid of *Agrobacterium tumefaciens* strain C58 with the *npt I* gene of Tn903, which confers resistance to Kanamycin. The entire *vir* region of the resulting plasmid, herein called pTiC58Δ, was left intact (Hepburn *et al.*, (1985) J. Gen. Microbiol. 131:2961-2969). The helper plasmid pTiC58Δ from strain Z707 was purified by cesium chloride gradient centrifugation and was electroporated into electrocompetent UIA143 cells. A transformant was selected on the basis of the pTiC58Δ plasmid-borne Kanamycin resistance gene and chromosomally-borne Erythromycin resistance gene, and the strain was named DA2569. Presence of pTiC58Δ in DA2569 was verified by PCR amplification using primers to detect selected vir gene regions and by Southern blot analysis of total DNA prepared from DA2569 candidate colonies probed with 32P-labeled DNA of pTiC58Δ purified by cesium chloride gradient centrifugation from cells of strain Z707.

EXAMPLE 5: Construction of a RecA-deficient *Agrobacterium* strain harboring the helper plasmid pMP90

Agrobacterium tumefaciens strain GV3101(pMP90) harbors a deleted version of pTiC58 called pMP90, from which the entire T-DNA region has been deleted and replaced with a gene conferring resistance to Gentamicin (Koncz and Schell, (1986) Mol. Gen. Genet. 204:383-396). DNA of plasmid pMP90 is prepared by methods such as cesium chloride gradient centrifugation or the MACHEREY-NAGEL NUCLEOBOND XTRA MAXI KIT "LOW COPY" (MACHEREY-NAGEL Inc.; Bethelem, PA) and is electroporated into UIA143 cells. A transformant is selected on the basis of the pMP90 plasmid-borne Gentamicin resistance gene (100 µg/mL) and the strain is named DAt20538. Presence of pMP90 in DAt20538 is verified by PCR amplification using primers to detect selected vir gene regions and by Southern blot analysis of total DNA prepared from DAt20538.

EXAMPLE 6: Construction of a RecA-deficient *Agrobacterium* strain harboring the helper plasmid pMP90RK

The helper plasmid pMP90 described in Example 5 was further modified by the introduction (via double crossover homologous recombination) of a 42 kbp *EcoR I* fragment derived from plasmid pRK2013 (Figurski and Helinski, (1979) Proc. Natl. Acad. Sci. USA 79:1648-1652). The 42 kbp fragment contains plasmid RK2-derived genes for plasmid replication and mobilization (*e.g. trfA, tra1, tra2, tra3,* and *oriT*), and a gene conferring resistance to Kanamycin. This manipulation replaced the Gentamicin resistance gene of plasmid pMP90, and the resulting plasmid was named pMP90RK (Koncz and Schell, *supra*). DNA of plasmid pMP90RK is prepared by methods such as cesium chloride gradient centrifugation or the MACHEREY-NAGEL NUCLEOBOND XTRA MAXI KIT "LOW COPY" and is electroporated into electrocompetent UIA143 cells. A transformant is selected on the basis of the pMP90RK plasmid-borne Kanamycin resistance gene and the strain is named DAt20539. Presence of pMP90RK in DAt20539 is verified by

PCR amplification using primers to detect selected vir gene regions and by Southern blot analysis of total DNA prepared from DAt20539.

EXAMPLE 7: Electroporation of pDAB9292 DNA into *Agrobacterium* strain DA2552

Electrocompetent DA2552 cells were prepared using a standard protocol (see Example 3). 50 µL of the competent DA2552 cells were thawed on ice and were transformed using 300 to 400 ng of plasmid pDAB9292 DNA. The DNA and cell mix was electroporated using prechilled electroporation cuvettes (0.2 cm) and a BIO-RAD GENE PULSER electroporator (BIO-RAD Inc.; Hercules, CA.) with the following conditions: Voltage: 2.5 kV, Pulse length: 5 msec, Capacitance output: 25 µFarad, Resistance: 200 ohms. After electroporation, 1 mL of YEP (gm/L: Yeast Extract 10, Peptone 10, NaCl 5) broth was added to the cuvette and the cell-YEP suspension was transferred to a 15 mL culture tube. The cells were incubated at 28° with gentle agitation for 4 hours after which the culture was plated on YEP + agar containing Kanamycin at 50 µg/mL and Erythromycin at 150 µg/mL. The plates were incubated for 2 to 4 days at 28° and colonies were selected and streaked onto fresh YEP + agar plates with antibiotics as above and incubated at 28° for 1 to 3 days. These colonies were verified as *Agrobacterium* using the ketolactose test (Bouzar *et al., (*1995) In: Methods in Molecular Biology (K. Gartland and M. Davey, eds.) *Agrobacterium* Protocols. (Vol. 44) HUMANA PRESS, Totowa, NJ. pp. 9-13. Several ketolactose positive colonies were selected to start 3 mL YEP (with antibiotics) seed cultures that were grown overnight at 28° while shaking. 300 µL of each seed culture was used to inoculate a 200 mL YEP (with antibiotics) overnight culture grown at 28° while shaking at 200 rpm. Plasmid DNA was prepared from 165 mL of each 200 mL overnight culture using a MACHEREY-NAGEL NUCLEOBOND® XTRA MAXI PLASMID DNA PURIFICATION kit. The manufacturer's protocol was followed, except 30 mL each of buffer RES, LYS, and NEU was used. The eluted DNA was stored at 4°.

Restriction enzyme digestion of the plasmid DNA with *BamH I* was used to validate the presence of pDAB9292 in these isolates, and colonies having the correct patterns were then further purified using two passages of single colony isolation. Plasmid DNA was

prepared from overnight cultures as described above and restriction digest analysis was used to verify the presence of the intact pDAB9292. Plasmid DNA of the pDAB9292 vector originally used in the DA2552 transformation was included as a digested standard. Four separate digest reactions (*Pst I*, *BamH I*, *Mfe I* and *Hind III*) were run using 750 ng to 1 µg of DNA. The reaction was allowed to run 1 to 2 hrs and was analyzed by agarose gel electrophoresis (0.8% w/v) and the DNA fragments were visualized by ethidium bromide staining. This *Agrobacterium* strain (*i.e.* DA2552 harboring pDAB9292) is named DAt13192. This strain provides the basis for a recombination-deficient "ternary" plant transformation system.

EXAMPLE 8: Electroporation of pDAB9292 DNA into *Agrobacterium* strain GV3101(pMP90)

Cells of *Agrobacterium tumefaciens* strain GV3101(pMP90) (Koncz and Schell, *supra*) were made electrocompetent by a standard protocol (see Example 3). 50 µL of the competent GV3101(pMP90) cells were thawed on ice and were transformed using 300 to 400 ng of plasmid pDAB9292 DNA. The DNA and cell mix was electroporated using prechilled electroporation cuvettes (0.2 cm) and a BIO-RAD GENE PULSER electroporator with the following conditions: Voltage: 2.5 kV, Pulse length: 5 msec, Capacitance output: 25 µFarad, Resistance: 200 ohms. After electroporation, 1 mL of YEP broth was added to the cuvette and the cell-YEP suspension was transferred to a 15 mL culture tube. The cells were incubated at 28° with gentle agitation for 4 hours after which the culture was plated on YEP + agar containing Kanamycin at 50 µg/mL and Gentamicin at 100 µg/mL. The plates were incubated for 2 to 4 days at 28° and colonies were selected and streaked onto fresh YEP + agar plates with antibiotics as above and incubated at 28° for 1 to 3 days. These colonies were verified as *Agrobacterium* using the ketolactose test. Several ketolactose positive colonies were selected to start 3 mL YEP (with antibiotics) seed cultures that were grown overnight at 28° while shaking. 300 µL of each seed culture was used to inoculate a 200 mL YEP (with antibiotics) overnight culture grown at 28° while shaking at 200 rpm. Plasmid DNA was prepared from 165 mL of each 200 mL overnight culture using a MACHEREY-NAGEL NUCLEOBOND® XTRA MAXI

PLASMID DNA PURIFICATION. The manufacturer's protocol was followed, except 30 mL each of buffer RES, LYS and NEU was used. The eluted DNA was stored at 4°.

Restriction enzyme digestion of the plasmid DNA with *BamH I* was used to validate the presence of pDAB9292 in these isolates, and colonies having the correct patterns were then further purified using two passages of single colony isolation. Plasmid DNA was prepared from overnight cultures as described above and restriction digest analysis was used to verify the presence of the intact pDAB9292. Plasmid DNA of the pDAB9292 vector originally used in the GV3101(pMP90) transformation was included as a digested standard. Four separate digest reactions (*Pst I*, *BamH I*, *Mfe I* and *Hind III*) were run using 750 ng to 1 µg of DNA. The reaction was allowed to run 1 to 2 hrs and was analyzed by agarose gel electrophoresis (0.8% w/v) and the DNA fragments were visualized by ethidium bromide staining. The *A. tumefaciens* GV3101 isolate harboring the pMP90 Ti helper plasmid and pDAB9292 is called DAt20712.

EXAMPLE 9: Electroporation of pDAB9292 DNA into *Agrobacterium* strain LBA4404

Cells of *Agrobacterium tumefaciens* strain LBA4404 (Ooms *et al.*, (1982) Plasmid 7:15-29) were made electrocompetent by a standard protocol (see Example 3). 50 µL of the competent LBA4404 cells were thawed on ice and were transformed using 300 to 400 ng of plasmid pDAB9292 DNA. The DNA and cell mix was electroporated using prechilled electroporation cuvettes (0.2 cm) and a BIO-RAD GENE PULSER electroporator with the following conditions: Voltage: 2.5 kV, Pulse length: 5 msec, Capacitance output: 25 µFarad, Resistance: 200 ohms. After electroporation, 1 mL of YEP broth was added to the cuvette and the cell-YEP suspension was transferred to a 15 mL culture tube. The cells were incubated at 28° with gentle agitation for 4 hours after which the culture was plated on YEP + agar containing Kanamycin at 50 µg/mL and Streptomycin at 250 µg/mL. The plates were incubated for 2 to 4 days at 28° and colonies were selected and streaked onto fresh YEP + agar plates with antibiotics as above and incubated at 28° for 1 to 3 days. These colonies were verified as *Agrobacterium* using the ketolactose test and were further purified using two passages of single colony isolation.

Several ketolactose positive colonies were selected to start 3 mL YEP (with antibiotics) seed cultures that were grown overnight at 28° while shaking. 300 µL of each seed culture was used to inoculate a 200 mL YEP (with antibiotics) overnight culture grown at 28° while shaking at 200 rpm. Plasmid DNA was prepared from 165 mL of each 200 mL overnight culture using a MACHEREY-NAGEL NUCLEOBOND® XTRA MAXI PLASMID DNA PURIFICATION kit. The manufacturer's protocol was followed, except 30 mL each of buffer RES, LYS and NEU was used. The eluted DNA was stored at 4°.

The presence of the intact pDAB9292 plasmid was verified by restriction digest analysis. Plasmid DNA of the pDAB9292 vector originally used in the LBA4404 transformation was included as a digested standard. Three separate digest reactions (*Pst I*, *BamH I*, and *Hind III*) were run using 750 ng to 1 µg of DNA. The reaction was allowed to run 1 to 2 hrs and was analyzed by agarose gel electrophoresis (0.8% w/v) and the DNA fragments were visualized by ethidium bromide staining. The *A. tumefaciens* LBA4404 isolate harboring pDAB9292 is called DAt20711. This strain provides the basis for a recombination-proficient "ternary" system.

EXAMPLE 10: Electroporation of pDAB9292 DNA into *Agrobacterium* strain DAt20538

Electrocompetent DAt20538 cells are prepared using a standard protocol (see Example 3). 50 µL of competent DAt20538 cells are thawed on ice and are transformed using 300 to 400 ng of plasmid pDAB9292 DNA. The DNA and cell mix is electroporated using prechilled electroporation cuvettes (0.2 cm) and a BIO-RAD GENE PULSER electroporator with the following conditions: Voltage: 2.5 kV, Pulse length: 5 msec, Capacitance output: 25 µFarad, Resistance: 200 ohms. After electroporation, 1 mL of YEP broth are added to the cuvette and the cell-YEP suspension is transferred to a 15 mL culture tube. The cells are incubated at 28° with gentle agitation for 4 hours after which the culture is plated on YEP + agar containing Kanamycin at 50 µg/mL and Gentamicin at 100 µg/mL. The plates are incubated for 2 to 4 days at 28° and colonies are selected and streaked onto fresh YEP + agar plates with antibiotics as above and incubated at 28° for 1 to 3 days. These colonies are verified as *Agrobacterium* using the ketolactose test and ketolactose positive colonies are further isolated using two passages of single colony isolation.

Colonies are selected to start 3 mL YEP (with antibiotics) seed cultures that are grown overnight at 28° while shaking. 300 µL of each seed culture is used to inoculate a 200 mL YEP (with antibiotics) overnight culture grown at 28° while shaking at 200 rpm. Plasmid DNA is prepared from 165 mL of each 200 mL overnight culture using a MACHEREY-NAGEL NUCLEOBOND® XTRA MAXI PLASMID DNA PURIFICATION kit. The manufacturer's protocol is followed, except 30 mL each of buffer RES, LYS and NEU are used. The eluted DNA is stored at 4°.

Restriction digest analysis is used to verify the presence of the intact pDAB9292 plasmid. Plasmid DNA of the pDAB9292 vector originally used in the DAt20538 transformation is included as a digested standard. Four separate digest reactions such as *Pst I*, *BamH I*, *Mfe I* and *Hind III* are run using 750 ng to 1 µg of DNA. The reaction is allowed to run 1 to 2 hrs and is analyzed by agarose gel electrophoresis (0.8% w/v) and the DNA fragments are visualized by ethidium bromide staining. The *A. tumefaciens* DAt20538 isolate harboring pDAB9292 is called DAt20538(pDAB9292).

EXAMPLE 11: Construction of plant transformation vectors having multiple repeated sequence elements and introduction into *Agrobacterium* strains

The utility of an engineered *Agrobacterium tumefaciens* strain having a deficiency in RecA function in combination with the auxiliary *vir* genes provided by the 14.8 *KpnI* VirBCDG fragment is illustrated herein. A binary plant transformation vector, pDAB101513 (Figure 4A), was constructed in *E. coli* cloning strain STBL2™ by a combination of standard cloning methods (as described, for example, in Sambrook *et al.*, (1989, *supra*) and Ausubel *et al.*, (1995, *supra*)) and GATEWAY™ technology (INVITROGEN). Binary vector pDAB101513 is based on the IncP-type replication origin of plasmid RK2, and the vector backbone harbors a bacterial gene conferring resistance to Spectinomycin (SpcR in Figure 4) at 100 µg/mL. The T-DNA border repeats are derived from the TL region of pTi15955. Within the Right Border (T-DNA Border B in Figure 4) and triple Left Borders (T-DNA Border A in Figure 4) of the T-DNA region of plasmid pDAB101513 are positioned 4 plant-expressible, plant-codon-optimized protein coding sequences (CDS), the transcription of each one being driven by a 1,991 bp maize ubiquitin1 promoter with associated intron1 (U.S. Patent No. 5,510,474). Three of the coding regions

encode separate *Bt* Cry1 proteins (Cry1Ca, SEQ ID NO:7; Cry1Fa, SEQ ID NO:9; and Cry1Ab, SEQ ID NO:11), each comprising around 3,500 bp. These coding regions were codon optimized for expression in maize plants using a maize (*Zea mays*) codon bias table calculated from analysis of 706 maize protein coding regions obtained from GENBANK deposits. Additional guidance regarding the design and production of synthetic genes can be found in, for example, WO 97/13402A1, U.S. Patent No. 6,166,302, and U.S. Patent No. 5,380,831. The three B.t protein coding regions are related to one another in the following fashion: The coding region for *cry1Ca* (SEQ ID NO:6) and the coding region for *cry1Fa* (SEQ ID NO:8) share 67% sequence homology; the coding regions for *cry1Ca* (SEQ ID NO:6) and *cry1Ab* (SEQ ID NO:10) share 69.5% sequence homology, and the coding regions for *cry1Fa* (SEQ ID NO:8) and *cry1Ab* (SEQ ID NO:10) share 67% sequence homology. Further, the C-terminal 1,600 bp of the CDS for *cry1Ca*, *cry1Fa,* and *cry1Ab* share 73% sequence homology. Each of these three coding regions is terminated by a 365 bp maize Per5 3' Untranslated Region (3'UTR) (U.S. Patent No. 6,384,207). The fourth gene comprises a plant-codon-optimized *aad1* coding region (SEQ ID NO:12) that encodes the AAD1 selectable marker protein (SEQ ID NO:13) (U.S. Patent No. 7,838,733) The *aad1* coding region is not related to the CDS for *cry1Ca*, *cry1Fa,* or *cry1Ab*. The coding region for *aad1* was designed using a plant-codon bias table. A maize codon bias table was calculated from 706 maize protein coding sequences obtained from sequences deposited in GENBANK. Codon usage tables for tobacco (*Nicotiana tabacum*, 1268 CDS), canola (*Brassica napus*, 530 CDS), cotton (*Gossypium hirsutum*, 197 CDS), and soybean (*Glycine max; ca.* 1000 CDS) were downloaded from data at the website http://www.kazusa.or.jp/codon/. A biased codon set that comprises frequently used codons common to both maize and dicot datasets, in appropriate rescaled average relative amounts, was calculated after omitting any redundant codon used less than about 10% of total codon uses for that amino acid in either plant type. The *aad1* gene is terminated by a maize Lipase 3'UTR (U.S. Patent No. 7,179,902). Thus, within the 22,729 bp T-DNA region of pDAB101513, the four copies of the maize ubi1 promoter comprise a total of 7,964 bases arranged in four direct repeats of almost 2 kbp (kilobase pairs) each, with each repeat being 100% related to the other. The three copies of the Per5 3'UTR comprise a total of 1,095 bases arranged in three direct repeat units, each one being 100% related to the other, and the

three coding regions *cry1Ca*, *cry1Fa,* and *cry1Ab* are arranged as direct repeats having between 67% and 73% homology to one another. In total, the T-region of pDAB101513 comprises about 86% highly repeated sequences, and may be conveniently illustrated below:

RB>Ubi1 promoter:*cry1Ca* CDS:Per5 3'UTR>Ubi1 promoter:*cry1Fa* CDS:Per5 3'UTR>Ubi1 promoter:*cry1Ab* CDS:Per5 3'UTR>Ubi1 promoter:*aad1* CDS:Lip 3'UTR>LB

The highly repeated nature of this construct required that the cloning steps be completed in the *E. coli* cloning strain STBL2™, which is specially engineered to maintain the integrity of clones containing such highly repeated DNA sequences.

Plasmid pDAB101513 was introduced by electroporation into electrocompetent cells of *A. tumefaciens* strain EHA105 (rendered Streptomycin resistant by virtue of a spontaneous chromosomal mutation), and Spectinomycin/Streptomycin-resistant isolates were verified by restriction digestion analysis to contain intact plasmid pDAB101513 prior to preparation of frozen glycerol stocks and storage at -80°. This strain is named EHA105(pDAB101513). Numerous individual cultures established from cells obtained from frozen glycerol stocks of EHA105(pDAB101513) were found to contain re-arranged or deleted versions of the pDAB101513 plasmid. For maize transformations, bulk cells of strain EHA105(pDAB101513) were harvested from an agar plate inoculated from a frozen glycerol stock and used directly as described in Example 13.

Plasmid pDAB101513 was successfully introduced by electroporation into electrocompetent cells of *A. tumefaciens* strain DA2552 (essentially a RecA-deficient version of strain EHA105) to produce strain DA2552(pDAB101513). Transformants selected by means of resistance to Erythromycin and Spectinomycin were validated by restriction enzyme digestion of plasmid DNA prior to preparation of frozen glycerol stocks and storage at -80°. Numerous individual cultures established from cells obtained from frozen glycerol stocks were found to contain intact pDAB101513 plasmid. Bulk cells of strain DA2552(pDAB101513) were harvested from an agar plate inoculated from a frozen glycerol stock and used for maize transformations (Example 13).

Plasmid pDAB101513 was successfully introduced by electroporation into electrocompetent cells of *A. tumefaciens* strain DAt13192 (strain DA2552 harboring

plasmid pDAB9292) to produce strain DAt13192(pDAB101513). Transformants selected by means of resistance to Erythromycin, Kanamycin, and Spectinomycin were validated by restriction enzyme digestion of plasmid DNA prior to preparation of frozen glycerol stocks and storage at -80°. Numerous individual cultures established from cells obtained from the frozen stocks were found to contain intact pDAB101513 plasmid. Bulk cells of strain DAt13192(pDAB101513) were harvested from an agar plate inoculated from a frozen glycerol stock and used for maize transformations (see Example 13).

In similar fashion, a derivative of pSB11 (the shuttle vector of the superbinary system) was constructed having a T-DNA region analogous to that of pDAB101513. Multiple attempts to construct a superbinary plasmid by standard methods in LBA4404(pSB1) were unsuccessful. All attempts resulted in isolation of highly rearranged and deleted pSB1-based cointegrant plasmids.

EXAMPLE 12: Construction of plant transformation vector pDAB101514 having multiple repeated sequence elements and introduction into *Agrobacterium* strains

The utility of an engineered *A. tumefaciens* strain having a deficiency in RecA function in combination with the auxiliary *vir* genes provided by the 14.8 *KpnI* VirBCDG fragment is further illustrated herein. A binary plant transformation vector, pDAB101514 (Figure 4B.), was constructed in *E. coli* cloning strain STBL2™ by a combination of standard cloning methods and GATEWAY™ technology. The structure of binary vector pDAB101514 is nearly the same as that of pDAB101513 (previous Example) with the exception of the expression elements used to drive expression of the *cry1Ca* gene. The transcription of the *cry1Ca* CDS in pDAB101514 is driven by a 1429 bp sugarcane bacilliform virus promoter (SCBV; Tzafrir *et al.,* (1998) Plant Molec. Biol. 38:347-356). The 5'UTR is comprised essentially of intron 6 of the maize alcohol dehydrogenase gene (GENBANK Accession X04049), flanked by 20 bases of exon 6 and 11 bases of exon 7. The transcription of this gene is terminated by a potato pinII 3'UTR (An *et al.*, (1989) Plant Cell. 1:115-122). The expression elements used to control expression of the *cry1Fa*, *cry1Ab*, and *aad1* genes are the same as were employed in pDAB101513. Thus, within the 22,586 bp T-DNA region of pDAB101514, the three copies of the maize ubi1 promoter comprise a total of 5,973 bases arranged in 3 direct repeats of almost 2 kbp each, with each

repeat being 100% related to the other. The two copies of the Per5 3'UTR comprise a total of 730 bases arranged in two direct repeat units, each one being 100% related to the other, and the three coding regions *cry1Ca*, *cry1Fa*, or *cry1Ab* are arranged as direct repeats having between 67% and 73% DNA sequence homology to one another. In total, the T-region of pDAB101514 comprises about 76% highly repeated DNA sequences, and the physical arrangement may be conveniently illustrated below:

RB>SCBV promoter:*cry1Ca* CDS:pinII 3'UTR>Ubi1 promoter:*cry1Fa* CDS:Per5 3'UTR>Ubi1 promoter:*cry1Ab* CDS:Per5 3'UTR>Ubi1 promoter:*aad1* CDS:Lip 3'UTR>LB

The highly repeated nature of this construct required that the cloning steps be completed in the *E. coli* cloning strain STBL2™, which is specially engineered to maintain the integrity of clones containing such highly repeated DNA sequences.

Plasmid pDAB101514 was introduced by electroporation into electrocompetent cells of *A. tumefaciens* strain EHA105 (rendered Streptomycin resistant by virtue of a spontaneous chromosomal mutation), and Spectinomycin/Streptomycin-resistant isolates were verified by restriction digestion analysis to contain intact plasmid pDAB101514 prior to preparation of frozen glycerol stocks and storage at -80°. This strain was named EHA105(pDAB101514). Numerous individual cultures established from EHA105(pDAB101514) cells obtained from frozen glycerol stocks were found to contain re-arranged or deleted versions of the pDAB101514 plasmid. For maize transformations, bulk cells of strain EHA105(pDAB101514) were harvested from an agar plate inoculated from a frozen glycerol stock and used by methods disclosed in Example 13.

Plasmid pDAB101514 was successfully introduced by electroporation into electrocompetent cells of *A. tumefaciens* strain DA2552 (essentially a RecA-deficient version of strain EHA105) to produce strain DA2552(pDAB101514). Transformants selected by means of resistance to Erythromycin and Spectinomycin were validated by restriction enzyme digestion of plasmid DNA prior to preparation of frozen glycerol stocks and storage at -80°. Numerous individual cultures established from cells obtained from frozen glycerol stocks were found to contain intact pDAB101514 plasmid. Bulk cells of

strain DA2552(pDAB101514) were harvested from an agar plate inoculated from a frozen glycerol stock and used for maize transformations by methods disclosed in Example 13.

Plasmid pDAB101514 was successfully introduced by electroporation into cells of *A. tumefaciens* strain DAt13192 (strain DA2552 harboring plasmid pDAB9292) to produce strain DAt13192(pDAB101514). Transformants selected by means of resistance to Erythromycin, Kanamycin, and Spectinomycin were validated by restriction enzyme digestion of plasmid DNA prior to preparation of frozen glycerol stocks and storage at -80°. Numerous individual cultures established from DAt13192(pDAB101514) cells obtained from the frozen stocks were found to contain intact pDAB101514 plasmid. Bulk cells of strain DAt13192(pDAB101514) were harvested from an agar plate inoculated from a frozen glycerol stock and used for maize transformations by methods disclosed in Example 13.

In similar fashion, a derivative of pSB11 (the shuttle vector of the superbinary system) was constructed having a T-DNA region analogous to that of pDAB101514. Multiple attempts to construct a superbinary plasmid by standard methods in LBA4404(pSB1) were unsuccessful. All attempts resulted in isolation of highly rearranged and deleted pSB1-based cointegrant plasmids.

EXAMPLE 13: Transformation of maize by *Agrobacterium* strains harboring binary vectors pDAB101513 and pDAB101514

Agrobacterium-Mediated Transformation of Maize Seeds from a Hi-II F1 cross (Armstrong *et al.*, (1991) Maize Genet. Coop. Newslett. 65:92-93) were planted into 5-gallon-pots containing a mixture of 95% METRO-MIX 360 soilless growing medium (SUN GRO HORTICULTURE; Bellevue, WA) and 5% clay/loam soil. The plants were grown in a greenhouse using a combination of high pressure sodium and metal halide lamps with a 16 hr light:8 hr dark photoperiod. Controlled sib-pollinations were performed to obtain immature F2 embryos for transformation. Maize ears were harvested at approximately 8-10 days post-pollination when immature embryos were between 1.0 mm and 2.0 mm in size.

Infection and co-cultivation. Maize ears were dehusked and surface sterilized by scrubbing with liquid soap, immersing in 20% commercial bleach (containing 5% sodium hypochlorite) for about 20 minutes, then rinsing three times with sterile water. A

suspension of *A. tumefaciens* cells harboring pDAB101513 or pDAB101514, binary vectors having three genes encoding the *Bt* Cry1Ca, Cry1Fa, and Cry1Ab proteins, and containing the *aad-1* plant selectable marker gene, was prepared by transferring 1 or 2 loops of bacteria (grown for 2-3 days at 28° on YEP agar medium containing appropriate antibiotics) into 5 mL of liquid infection medium (LS Basal Medium (Linsmaier and Skoog, (1965) Physiologia Plantarum 18:100–127), N6 vitamins (Chu *et al.*, (1975) Scientia Sinica 18:659-668), 1.5 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D), 68.5 gm/L sucrose, 36.0 gm/L glucose, 6 mM L-proline, pH 5.2] containing 200 µM acetosyringone. The solution was vortexed until a uniform suspension was achieved, and the concentration was adjusted to a final optical density of approximately 0.4 at 550 nm.

Immature embryos were isolated directly into a microcentrifuge tube containing 2 mL of the infection medium. The medium was removed and replaced with 1 mL of the *Agrobacterium* solution and the *Agrobacterium*/embryo solution was incubated for 5 to 10 minutes at room temperature. Embryos were then transferred to cocultivation medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 30.0 gm/L sucrose, 6 mM L-proline, 0.85 mg/L AgNO3, 2.8 gm/L GELLAN GUM™ (*PHYTO*TECHNOLOGY LABORATORIES; Lenexa, KS), pH 5.8) containing 200 µM acetosyringone and cocultivated for 3-4 days at 20° in the dark.

After cocultivation, the embryos were transferred to resting medium containing MS salts and vitamins (Frame *et al.*, 2011, *Genetic Transformation Using Maize Immature Zygotic Embryos*. In: Plant Embryo Culture Methods and Protocols: Methods in Molecular Biology. T. A. Thorpe and E. C. Yeung, (Eds), SPRINGER SCIENCE AND BUSINESS MEDIA, LLC. pp 327-341), 6 mM L-proline, 100 mg/L myo-inositol, 500 mg/L MES (2-(N-morpholino) ethanesulfonic acid monohydrate; *PHYTO*TECHNOLOGIES LABR.), 30 gm/L sucrose, 1.5 mg/L 2,4-D, 0.85 $AgNO_3$, 250 mg/L Cefotaxime, 2.8 gm/L GELLAN GUM™, pH 5.8. Approximately 7 days later, embryos were transferred to the same medium supplemented with 100 nM Haloxyfop. Transformed isolates were identified after approximately 8 weeks and were bulked up by transferring to fresh selection medium at 2-week intervals for regeneration and analysis.

Regeneration and seed production. For regeneration, the cultures were transferred to "28" induction medium (MS salts and vitamins, 30 gm/L sucrose, 5 mg/L

Benzylaminopurine, 0.25 mg/L 2, 4-D, 250 mg/L Cefotaxime, 2.5 gm/L GELLAN GUM™, pH 5.7) supplemented with 100 nM Haloxyfop. Incubation was for 1 week under low-light conditions (14 $\mu Em^{-2}s^{-1}$), then 1 week under high-light conditions (approximately 89 $\mu Em^{-2}s^{-1}$). Tissues were subsequently transferred to "36" regeneration medium (same as induction medium except lacking plant growth regulators). When plantlets were 3 cm to 5 cm in length, they were transferred to glass culture tubes containing SHGA medium [(Schenk and Hildebrandt salts and vitamins, *PHYTO*TECHNOLOGIES LABR.), 1.0 gm/L myo-inositol, 10 gm/L sucrose and 2.0 gm/L GELLAN GUM™, pH 5.8] to allow for further growth and development of the shoot and roots. Plants were transplanted to the same soil mixture as described earlier and grown to flowering in the greenhouse. Samples of plant tissues were harvested and used in insect bioassays by methods disclosed in Example 14 and for molecular and biochemical analyses. Controlled pollinations for seed production are conducted.

Those skilled in the art of maize transformation will understand that other methods are available for maize transformation and for selection of transformed plants when other plant expressible selectable marker genes (*e.g.* herbicide tolerance genes) are used.

EXAMPLE 14: *In vitro* bioassays of leaf samples against maize insect pests

The lepidopteran species assayed were the corn earworm (CEW; *Helicoverpa zea* (Boddie)), European corn borer, (ECB; *Ostrinia nubilalis* (Hübner)), and fall armyworm (FAW; *Spodoptera frugiperda* (J.E. Smith)). Eggs for these insects were obtained from BENZON RESEARCH (Carlisle, PA).

First Tier Bioassay: High-throughput 96-well Bioassay 96-well trays (TPP-US; St. Louis, MO) were partially filled with a 2% agar solution (SIGMA-ALDRICH) and agar was allowed to solidify. Using a standard hand-held paper punch, three 1/8 inch diameter leaf discs were sampled for each of the two insect species (CEW and FAW) tested in this format. One leaf disc was placed in a single well of the 96-well plate; there were three plates for each insect tested (one for each replicate/leaf disc). An egg-seeding device was used to administer insect eggs into each well of the 96-well plate. Plates were then sealed with perforated sticky lids and also enclosed with the plastic lid that accompanies the plates. Plates were held at 30°, 40% Relative Humidity (RH), 16 hours light: 8 hours dark

for three days. Grading was conducted using a 0 – 1 – 2 scale, in which 0 indicated < 25% leaf disc damage, 1 indicated 25-50% leaf disc damage, and 2 indicated >50% leaf disc damage within each well. Damage scores for each test were averaged and used alongside protein expression analysis to conduct correlation analyses. Plants whose average insect damage score was 0.67 or less were considered active against the tested pest.

Second Tier Bioassay: 32-well Bioassay 32-well trays (C-D INTERNATIONAL; Pitman, NJ) were partially filled with a 2% agar solution and agar was allowed to solidify. Leaf sections approximately 1 inch square were taken from each plant and placed singly into wells of the 32-well trays. One leaf piece was placed into each well, and two leaf pieces were tested per plant and per insect. Insects (ECB and FAW) were mass-infested using a paintbrush, placing 10-20 neonate larvae into each well. Trays were sealed with perforated sticky lids which allowed ventilation during the test. Trays were placed at 28°, 40% RH, 16 hours light: 8 hours dark for three days. After the duration of the test, a simple percent damage score was taken for each leaf piece. Damage scores for each test were averaged and used alongside protein expression analysis to conduct correlation analyses. Plants whose average insect damage ratings were 25% or less were considered active against the tested pest.

Statistical Analysis All analyses were conducted in JMP 8.0.2 (SAS INSTITUTE Inc., Cary, North Carolina). One-way ANOVA analysis was used to determine significant differences between the treatments and the negative control plants for insect damage data. The Tukey-Kramer HSD comparison of means was also used to further evaluate significant differences among the treatments. In addition, linear regression (least fit squares) analysis was used to correlate quantitative protein expression with insect activity measurements.

Bioassay results are summarized in Table 2.

EXAMPLE 15: Biochemical and molecular characterization of maize tissues transformed with pDAB101513

Multiple transformation experiments were performed with engineered *A. tumefaciens* strains EHA105(pDAB101513), DA2552(pDAB101513) and DAt13192(pDAB101513). Copy numbers of the four transgenes in transgenic T_0 plants were estimated by hydrolysis probe assays (Bubner and Baldwin, (2004) Plant Cell Rep. 23:263-271) using gene-specific

oligonucleotides. Protein extracts from plants with 1 to 3 copies ("Low Copy") of the genes were further examined for production of the *Bt* Cry1Ca, Cry1Fa, and Cry1Ab proteins, and for the AAD1 protein, by ELISA methods using commercially-produced antibody kits (ENVIROLOGIX™, Portland, MA). Some plants were found that produced all four proteins (Table 3). In addition, leaf pieces from the plants were bioassayed for activity against three maize insect pests: corn earworm (CEW, *Helicoverpa zea*), fall armyworm (FAW, *Spodoptera frugiperda*) and European corn borer (ECB, *Ostrinia nubilalis*) in feeding assays (EXAMPLE 14). Some plants were found that had all four transgenes in low copy number, produced all four proteins, and had insect activity against all three pests (Table 4). No transformed plants meeting these criteria were obtained from experiments using the EHA105(pDAB101513) or DA2552(pDAB101513) strains (Table 4). Thus, a feature of strain DAt13192, comprising a deletion of the chromosomal *recA* gene, further comprising a full set of pTiBo542-derived *vir* genes harbored on pTiEHA105, and even further comprising a partial set of pTiBo542-derived *vir* genes harbored on the 14.8 *KpnI* VirBCDG fragment of pDAB9292, is that it is able to efficiently produce transformed maize plants with large T-DNA regions comprised of highly repeated sequence elements.

EXAMPLE 16: Biochemical and molecular characterization of maize tissues transformed with pDAB101514

Multiple transformation experiments were performed with engineered *A. tumefaciens* strains EHA105(pDAB101514), DA2552(pDAB101514), and DAt13192(pDAB101514). Copy numbers of the four transgenes in transgenic T_0 plants were estimated by hydrolysis probe assays (Bubner and Baldwin, *supra*) using gene-specific oligonucleotides. Protein extracts from plants with 1 to 3 copies ("Low Copy") of the genes were further examined for production of the *Bt* Cry1Ca, Cry1Fa, and Cry1Ab proteins, and for the AAD1 protein, by ELISA methods using commercially-produced antibody kits (ENVIROLOGIX™, Portland, MA). In addition, leaf pieces from the plants were bioassayed for activity against three maize insect pests in feeding assays (Example 14). Some plants were found that had all four transgenes in low copy number, produced all four proteins, and had insect activity against all three pests (Table 5). No transformed plants meeting these criteria were obtained from experiments using the

EHA105(pDAB101514) or DA2552(pDAB101514) strains. Thus, a feature of strain DAt13192, comprising a deletion of the chromosomal *recA* gene, further comprising a full set of pTiBo542-derived *vir* genes harbored on pTiEHA105, and even further comprising a partial set of pTiBo542-derived *vir* genes harbored on the 14.8 *KpnI* VirBCDG fragment of pDAB9292, is that it is able to efficiently produce transformed maize plants with large T-DNA regions comprised of highly repeated sequence elements.

EXAMPLE 17: Identification and characterization of a neutral integration site in the *Agrobacterium tumefaciens* LBA4404 chromosome

The plant-inducible *picA/pgl* locus of the *A. tumefaciens* strain C58 chromosome (GENBANK Accession AE0009243) was identified as a non-essential gene into which DNA fragments could be integrated (Rong *et al.,* (1990) J. Bacteriol. 172:5828–5836; Rong *et al*., (1991) J. Bacteriol. 173:5110–5120). A similar neutral integration site in the genome of *A. tumefaciens* strain LBA4404 has not been reported. We describe here the identification and sequencing of a genomic region of LBA4404 that includes sequences partially homologous to the C58 *picA/pgl* locus. Cells of LBA4404 (INVITROGEN) were grown in YM medium (gm/L: yeast extract, 0.4; mannitol, 10; NaCl, 0.1; $MgSO_4$ $7H_2O$, 0.2; K_2HPO_4 $3H_2O$, 0.5) at 30° overnight. Genomic DNA was prepared from a 1 mL culture using the EASY DNA kit (INVITROGEN) according to the manufacturer's protocols. Degenerate primers were designed based upon two regions of homology between the C58 PicA protein sequence and homologues identified from *Arabidopsis thaliana*, *Caldicellulosiruptor saccharolyticus, Alkaliphilus metalliredigenes,* and *Clostridium acetobutylicum*. LBA4404 genomic DNA was used as a template for the polymerase chain reaction (PCR) using HERCULASE™ MASTER MIX (STRATAGENE; San Diego, CA) and degenerate primers At*nilA*1Fa (5'-GACAGTCCNAATACSGAYGG-3'; SEQ ID NO:14; corresponding to amino acids 273-279 of the C58 PicA protein) and At*nilA*3R (5'-GTYTTSAGNCGSAGSCCSCGRTCSGT-3'; SEQ ID NO:15, corresponding to the complementary strand coding for amino acids 364-369 of the C58 PicA protein). Thermocycling conditions used were: 1 cycle of 94°, 2 min; 25 cycles of [94°, 30 sec; 55°, 30 sec; 72°, 60 sec]; 1 cycle of 72°, 7 min. Degenerate nucleotide designations in the

primer sequences correspond to DNA nucleotides as follows: N = A, C, G, or T; Y = T or C; R = A or G; and S = C or G. A 285 base pair (bp) product was isolated, cloned into the vector pCR2.1-TOPO (INVITROGEN) in *Escherichia coli* TOP10 cells (INVITROGEN), and the DNA sequence was determined. The sequence was found to be homologous, but not identical, to a region of the C58 *picA* gene (85% sequence identity), and the LBA4404 genomic region that it represents is referred to herein as the *nilA* fragment.

Additional primers complementary to the 285 bp LBA4404 *nilA* fragment were designed to be used as anchors for PCR amplification of genomic fragments flanking both ends of the 285 bp sequence. These were paired in the PCR reactions with primers designed from sequences of the flanking regions of the C58 *picA* gene. Sequences of amplified fragments originating from within the 285 bp sequence and extending into both *nilA* fragment flanking regions were determined and used to design other primers for subsequent PCR reactions. Using LBA4404 genomic DNA template with primers *nilA*2F (5'- CCATCCTCATAACACCAGCT-3'; SEQ ID NO:16) and *nilA*2R (5'- GCAGATCATCGATACGACCA-3'; SEQ ID NO:17), an approximately 2 kilobase (kbp) PCR fragment was generated and cloned into pCR®-BLUNT II/XL-TOPO® using the TOPO TA cloning kit (INVITROGEN) to produce plasmid pDOW3719 (Figure 2). Sequence analysis of the insert fragment of pDOW3719 yielded an 1,796 bp sequence (SEQ ID NO:18) which comprises a longest open reading frame (ORF) that encodes a putative protein of 531 amino acids. A shorter ORF in the same reading frame encodes a putative protein of 523 amino acids. The LBA4404 523 amino acid putative protein shows 88% similarity, 85% identity with the C58 PicA protein. The coding sequences for the LBA4404 523 amino acid putative protein and the C58 PicA protein have 81% identity. Thus, the *nilA* fragment of LBA4404 represents a genomic segment that includes a putative gene that is substantially diverged from the C58 *picA* gene. In this disclosure, the 1.8 kbp genomic sequence represented by SEQ ID NO:18 is referred to as the *nilA* locus.

Plasmid pDOW3719, having the colE1 origin of replication, is not expected to replicate autonomously in *A. tumefaciens* cells. DNA of plasmid pDOW3719 was used to transform cells of *A. tumefaciens* strain LBA4404 by electroporation. Selection for Kanamycin resistance (harbored on pDOW3719) identified transformants that had integrated pDOW3719 into the chromosome of LBA4404 via recombination mediated by

the 1.8 kbp homology regions present in the LBA4404 chromosome and on pDOW3719. Such an integration event results in the creation of a linear copy of the pDOW3719 vector plasmid sequence flanked on each side by the now-duplicated 1.8 kbp homology region. Kanamycin resistant LBA4404 transformants were isolated and screened for insertion of pDOW3719 by PCR analysis. Genomic DNA preparations of the transformants were used as template in PCR reactions with 5 primers sets: *i*) M13F primer paired with M13R primer, which flank the insert in pDOW3719, *ii*) M13F primer paired with primer AS4R (comprising bases complementary to residues 1041 to 1060 of SEQ ID NO:18), *iii*) M13F primer paired with primer AS10R (comprising bases complementary to residues 1,320 to 1,337 of SEQ ID NO:18), *iv*) M13F primer paired with primer AS11R (comprising bases complementary to residues 1,391 to 1,406 of SEQ ID NO:18), and *v*) M13R primer paired with primer AS9F (comprising bases 634 to 649 of SEQ ID NO:18). In control reactions, all of these primer sets amplified expected sized fragments when pDOW3719 plasmid DNA was used as template. However, when genomic DNA from a Kanamycin resistant LBA4404 transformant was used as template, PCR using the M13F and M13R primer pair did not yield amplified products, indicating that no intact (non-integrated) pDOW3719 plasmid DNA was co-purified with the genomic DNA. PCR analysis of the genomic DNA samples with the other four primer pairs showed production of expected sized DNA fragments. These results indicate that the Kanamycin resistance of the LBA4404 transformants is conferred by pDOW3719 DNA which has integrated into the genome.

One such transformant [LBA4404*nilA*-int1] was used to test the effect that the genomic insertion into the *nilA* locus has on the ability of the strain to transform *Arabidopsis thaliana*. Binary vector pDAB3779, which contains a plant expressible gene encoding the PAT protein (which confers resistance to the herbicide BASTA™) was transformed into cells of strains LBA4404*nilA*-int1 and LBA4404, with selection for Spectinomycin resistance. These strains were then used to conduct *Arabidopsis* transformation experiments using the methods of Weigel and Glazebrook (*supra*). No difference was seen in the transformation frequencies obtained with the two strains. Thus, a feature of the embodiments and methods described herein is that insertion of a foreign DNA fragment into the chromosomal *nilA* locus of *A. tumefaciens* strain LBA4404 that comprises

SEQ ID NO:18 has no effect on the growth or plant transformation capability of such engineered strain.

EXAMPLE 18: Construction of a suicide derivative of pDOW3719 for integration into the LBA4404 *nilA* locus

The insertion of a multiple cloning site in the *nilA* locus cloned in pDOW3719 was accomplished by splice overlap extension (SOE) PCR (Horton *et al.*, (1990) BioTechniques 8:528-535). SOE PCR reactions were carried out using HERCULASE™ master mix according to the manufacturer's protocols. A portion of the *nilA* locus was amplified using pDOW3719 DNA as template with primer *nilA*5' (5'-CCGGCTCTTCCAGCTCCTCATGCACGAACAACGAGAAACGAGC-3'; SEQ ID NO:19) paired with primer *nilA*_MCS_SOER (5'-GAATGGTGAAACCTCTAGATTAATTAA GGATCCCCGGGTACCGAAAAGCCCGACATTGC-3'; SEQ ID NO:20) to produce an approximately 800 bp fragment. A second portion of the *nilA* locus was amplified using pDOW3719 DNA as template and primer *nilA*_MCS_SOEF (5'-GCAATGTCGGGCTTTTCGG TACCCGGGGATCCTTAATTAATCTAGAGGTTTCACCATTC-3'; SEQ ID NO:21) paired with primer *nilA*3' (5'-GGAATTCTCAGTGGCTTTCATGGGTTTTCTCG-3'; SEQ ID NO:22) to produce an approximately 900 bp fragment. The resulting fragments were then gel purified (NUCLEOSPIN™, CLONTECH; Mountain View, CA), and used as template for amplification with primers *nilA*5' and *nilA*3' to yield a 1.6 kbp fragment, which sequence is disclosed as SEQ ID NO:23 (*nilA* MCS). The resultant fragment was digested with *Pvu I* and *Sap I* (NEB) and ligated to pBCSK+sacBI DNA (INVITROGEN) digested with the same restriction enzymes, using T4 DNA ligase (NEB). *E. coli* TOP10 cells were transformed with the ligation mixture, and transformants were selected on LB soy agar (TEKNOVA; Hollister, CA) supplemented with 30 µg/mL Chloramphenicol. Clones were screened by restriction digestion with *Pvu I* and *Kpn I* (NEB). The *nilA* locus region of positive clones was sequence verified, and the resulting plasmid was named pDOW3721. A feature of pDOW3721 is that a multiple cloning site (MCS) containing recognition sequences for restriction enzymes *Sph I*, *Kpn I*, *Sma I*, *BamH I*, *Pac I*, *Ase I* and *Xba I* is

flanked on one side by 852 bp of LBA4404-derived bases, and on the other side by 745 bp of LBA4404-derived bases.

Thus, a foreign DNA fragment may be cloned into the MCS of pDOW3721, and thence integrated into the LBA4404 chromosomal *nilA* locus by virtue of homologous recombination mediated by the LBA4404-derived flanking sequences. Single crossover events, by means of which the entire pDOW3721 plasmid sequence is integrated into the LBA4404 chromosome, may be resolved into double crossover events by counterselection on sucrose containing media. On such media, the sucrose is converted to a toxic product upon enzymolysis by the SacB protein encoded by the *sacB* gene (Reid and Collmer, (1987) Gene 57:239-246; Quandt *et al.*, (1993) Gene 127:15–21). Thus, transformants able to survive on sucrose-containing growth medium will have undergone a second crossover event that eliminates the pDOW3721 plasmid vector backbone from the chromosome, leaving behind the disrupted *nilA* locus containing the integrated foreign DNA fragment. Many reports have shown for the last 10 years that the transfer of vector backbone sequences is quite common. The ratio of the plants that acquired the backbone sequences in transformants ranged typically between 20% and 50%, and was sometimes as high as 75% or more.

As one exemplification of the utility of pDOW3721, the 14.8 *KpnI* VirBCDG fragment was prepared from plasmid pSB1 and ligated to *Kpn I* digested pDOW3721 DNA, using T4 DNA ligase. *E. coli* TOP10 cells were transformed with the ligation mixture, and transformants were selected on LB soy agar supplemented with 30 µg/mL Chloramphenicol. Clones were screened by restriction digestion with *EcoR I* and *Hind III*. The resultant plasmid was named pDOW3722.

EXAMPLE 19: Identification of nucleotide sequences upstream and downstream of *nilA*

The LBA4404*nilA*-int1 strain of *A. tumefaciens*, containing a genomic integration of plasmid pDOW3719 (Figure 2 and Example 18), was used to identify additional sequences positioned upstream and downstream of the *nilA* genomic region. pDOW3719 contains a 1,796 bp PCR amplicon of the *A. tumefaciens* strain LBA4404 *nilA* locus cloned into PCR-BLUNT II/XL-TOPO® (INVITROGEN). This plasmid was integrated into the genome of

A. tumefaciens strain LBA4404 via homologous recombination. Colonies which contained the integrated plasmid were identified by resistance to Kanamycin (Example 17). The integrated plasmid, and the elements contained within it, may be used as tools for the isolation and characterization of additional nucleotide sequences via a "plasmid rescue" technique.

Genomic DNA (gDNA) was prepared from cells of the LBA4404*nilA*-int1 strain by a protocol for bacterial genomic DNA isolation (Sambrook *et al., supra*). One microgram of gDNA was individually digested with the following enzymes (all obtained from NEB): *Hind III*, *BamH I*, *Pst I*, *Asc I*, and *Sac II*. These restriction enzymes were chosen specifically to produce gDNA fragments that map upstream and downstream of the *nilA* locus. The *Hind III*, *BamH I*, and *Pst I* restriction enzymes were selected because their recognition sites are unique within the pDOW3719 sequence. Moreover, these enzyme recognition sites are located at the junctions between the *nilA* locus amplicon fragment and the PCR-BLUNT II/XL-TOPO® vector (Figure 2). Cleavage of gDNA with these enzymes and self ligation of the resulting fragments thus results in a plasmid rescue fragment which contains the uncharacterized genomic sequences ligated adjacent to the M13 forward universal primer or the M13 reverse universal primer binding sites of the pDOW3917 plasmid. Such clones are isolated by transforming the ligation mixture into *E. coli* cells, with selection for the Kanamycin resistance gene harbored by pDOW3917.

Further, the pDOW3719 plasmid does not contain recognition sites for the *Asc I* and *Sac II* restriction enzymes. Therefore, gDNA fragments generated by these restriction enzymes would produce a chimeric DNA fragment which spans the entire length of the integrated pDOW3719 plasmid sequence and includes the gDNA regions which flank both sides of the integrated pDOW3719 plasmid.

The gDNA fragments which resulted from restriction enzyme digestion as described above were self-ligated using T4 Ligase (ROCHE APPLIED SCIENCES; Indianapolis, IN). The ligation products were transformed into *E. coli* ONESHOT® TOP10 CELLS (INVITROGEN) and plated on LB media containing Kanamycin (50 µg/mL). Individual colonies were selected and plasmid DNA was isolated and characterized via plasmid restriction enzyme digestion patterns. Clones which contained plasmids exhibiting a

consistent restriction enzyme digestion banding pattern as compared to one another were advanced for use in sequencing reactions.

The nucleotide sequences of the gDNA upstream and downstream from the *nilA* locus were determined using a "genome walking" technique. Sequencing primers corresponding to the known gDNA sequence (as present in pDOW3719) were designed and used with the CEQ™ DYE TERMINATOR CYCLE SEQUENCING KIT according to the manufacturer's recommendations (BECKMAN COULTER; Fullerton, CA). From the determined sequence, a second set of primers, located in previously unknown genomic sequence, was designed and used to generate additional sequencing data. This process was repeated until all of the available gDNA nucleotide sequence was determined. This technique generated 2,936 bp of sequence upstream from the *nilA* locus, and 4,361 bp of sequence downstream from the *nilA* locus. In combination with the 1,796 bp of the previously identified *nilA* locus, the newly identified upstream and downstream flanking sequences regions netted a 9,093 bp sequence comprising the *nilA* genomic region (SEQ ID NO:24), which extends in both directions from the originally identified *nilA* locus.

EXAMPLE 20: Construction of a vector for integration into the LBA4404 *nilA* genomic region

An integration vector for homology-mediated integration of foreign DNA sequences into the *A. tumefaciens* LBA4404 *nilA* genomic region was designed and constructed. A 6.3 kbp fragment of the *nilA* genomic region spanning the *nilA* locus was PCR amplified using the FAILSAFE™ PCR KIT (EPICENTRE®, Madison, WI). The amplified fragment was ligated into the PCR®8/GW/TOPO® vector (INVITROGEN), and positive clones were confirmed via restriction enzyme digestion and DNA sequence verification. The resulting vector, pDAB9615, was further modified by the addition of an oligonucleotide fragment containing multiple unique restriction enzyme recognition sites. These restriction sites, flanked by 3,244 bp and 3,128 bp regions of the *nilA* genomic region, serve as cloning sites for the introduction of foreign nucleotide sequences. The resulting vector was named pDAB9618 (Figure 3).

A 15,549 bp fragment containing the 14.8 *KpnI* VirBCDG fragment of pSB1 and a bacterial Kanamycin resistance gene was prepared by digestion of pDAB9292 DNA with

Kpn I plus *Bst1107 I.* This fragment was then ligated to DNA of pDAB9618 that had been digested with *Kpn I* plus *Swa I,* to produce vector pDAB9621 (Figure 3). The GATEWAY® reaction was then used to move the portion of pDAB9621 containing the 14.8 *KpnI* VirBCDG fragment and bacterial Kanamycin resistance gene, flanked on each side by 3 kbp of LBA4404 *nilA* genomic region sequences, into the GATEWAY® PDEST™14 vector via AN L-R CLONASE® reaction (INVITROGEN). The resulting plasmid, pDAB9698 (Figure 3, SEQ ID NO:25), was confirmed via restriction enzyme digestion and DNA sequencing reactions. pDAB9698 served as an integration vector for integrating the pTiBo542-derived *vir* genes from pSB1 (harbored on the 14.8 *KpnI* VirBCDG fragment) into the *nilA* chromosomal region of *A. tumefaciens* strain LBA4404.

EXAMPLE 21: Chromosomal integration of the 14.8 *KpnI* VirBCDG fragment via homologous recombination

DNA of plasmid pDAB9698 was produced using a NUCLEOBOND® AX ANION EXCHANGE CHROMATOGRAPHY PLASMID DNA ISOLATION KIT (MACHEREY-NAGEL). The purified plasmid DNA was electroporated into *A. tumefaciens* LBA4404 CELLS (INVITROGEN). Briefly, 500 ng of plasmid DNA was incubated with the cells at 4° for 10 minutes. This mixture was pipetted into an ice-chilled 0.2 cm GENE PULSER® CUVETTE (BIO-RAD) and electroporated using the BIO-RAD GENE PULSER with the following settings: capacitance output 25 µFarad, capacitance extender 960 µFarad, resistance 200 ohms, and voltage 2.5 kVolts. Immediately after electroporation, 950 µL of SOC medium (INVITROGEN) was added and the mixture was transferred to a Falcon 2059 tube (BECTON DICKINSON AND CO.; Franklin Lakes, NJ). The transformed cells were then incubated at 28° for 5 to 6 hours. After incubation, the cells were plated on separate YEP medium plates containing Kanamycin (50 µg/mL). The plates were grown inverted at 28° for 36 to 48 hours. Single colonies were picked and propagated in 5 mL of liquid YEP containing Kanamycin (50 µg/mL) for approximately 36 hours at 28°. These cultures were used to prepare glycerol stock cultures by vigorous mixing with an equal volume of 100% sterile glycerol, followed by freezing and storage at -80°.

EXAMPLE 22: Phenotypic and molecular confirmation of the chromosomal integration of the 14.8 *KpnI* VirBCDG fragment

pDAB9698, having the colE1 origin of replication, is not expected to replicate autonomously in *A. tumefaciens* cells. Thus, upon transformation of pDAB9698 DNA into LBA4404 cells, stable Kanamycin resistance results from the integration of DNA of pDAB9698 into autonomously replicating *Agrobacterium* genetic elements. These plasmid integrants will fall into four classes that can be used according to various embodiments of the *Agrobacterium* strains and methods for their use as described herein. The first class comprises cells in which pDAB9698 DNA has integrated into a site remote from the *nilA* genomic region by means of nonhomologous recombination. These cells should be Kanamycin resistant by virtue of the Kanamycin resistance gene adjacent to the 14.8 *KpnI* VirBCDG fragment, and additionally should be resistant to Ampicillin by virtue of the Ampicillin resistance gene harbored on the pDAB9698 backbone vector (pDEST™14). The second class comprises cells in which the pDAB9698 DNA has integrated into the autonomously-replicating pAL4404 Ti helper plasmid (natively resident in LBA4404) by virtue of homologous recombination mediated by the pTiBo542-derived VirBCDG genes present on pDAB9698 and the pTiACH5-derived VirBCDG genes present on pAL4404. These cells should also be resistant to both Kanamycin and Ampicillin. The third class comprises cells in which pDAB9698 DNA has integrated into the LBA4404 *nilA* genomic region by virtue of a single homologous recombination (crossover) event mediated by either of the approximately 3 kbp *nilA* genomic region sequences harbored on pDAB9698, and which flank the 15,549 bp fragment containing the 14.8 *KpnI* VirBCDG fragment of pSB1 and the Kanamycin resistance gene. These cells should also be resistant to both Kanamycin and Ampicillin. The fourth class comprises cells in which the single crossover event of class 3 cells above undergoes a second crossover event mediated by the now-duplicated 3 kbp *nilA* genomic region sequences that are generated as a consequence of the single crossover event. Depending upon which of the flanking 3 kbp *nilA* genomic region sequences generated the single crossover event, and which of these flanking sequences generates the second crossover event, the resultant cells should either be Kanamycin sensitive, and Ampicillin resistant, or Kanamycin resistant and Ampicillin sensitive. Preferred cells as described herein comprise the latter class, that is, cells that are

Kanamycin resistant and Ampicillin sensitive. These double crossover events, which do not contain the pDEST™14 plasmid backbone, are desirable as they do not contain superfluous genetic elements such as the colE1 replication origin and Ampicillin resistance gene.

Putative transformants isolated in Example 21 were screened for a desirable double homologous recombination-mediated integration event. Kanamycin-resistant isolates having the 15,549 bp fragment containing the 14.8 *KpnI* VirBCDG fragment of pSB1 and the Kanamycin resistance gene (and lacking the pDEST™14 vector backbone) were identified via sensitivity to Ampicillin. The putative transformants were grown in 3 mL of YEP containing Kanamycin (50 µg/mL) at 28° for approximately 36 hours. These cultures were then streaked onto solid YEP media containing various single antibiotics as follows (concentrations in µg/mL): Rifampicin, 100; Kanamycin, 50; Streptomycin, 125; Chloramphenicol, 50; Erythromycin, 200; Tetracycline, 12.5; and Ampicillin, 100. The plates were incubated at 28° for 48 hours and colony growth was scored. A strain was identified that was resistant to Kanamycin, Rifampcin (chromosomal marker), and Streptomycin (pAL4404 marker; Ooms *et al*., supra). Moreover, the strain was sensitive to Chloramphenicol, Erythromycin, and Tetracycline. Most significantly, the strain was sensitive to Ampicillin. This drug screen phenotype is indicative of a desirable double crossover homologous recombination event, wherein the 15,549 bp fragment containing the 14.8 *KpnI* VirBCDG fragment of pSB1 and the Kanamycin resistance gene are integrated into the *A. tumefaciens* LBA4404 chromosome. This strain is called DAt16174.

The presence of the pTiBo542-derived VirBCDG genes in strain DAt16174 was further confirmed by molecular characterization. Genomic DNA of strain DAt16174 was isolated using a bacterial genomic DNA isolation protocol (Sambrook *et al.*, *supra* and updates thereof). PCR primers were designed to amplify overlapping fragments of the chromosomally integrated VirBCDG genes. PCR reactions using the primers described in Table 6 were completed using the FAILSAFE™ PCR KIT (EPICENTRE®) per the manufacturer's directions. Due to the large total molecular size of the integrated VirBCDG genes, the amplifications were done to produce five overlapping fragments. Amplicons of the expected size were purified from agarose gels using the QIAEX II GEL EXTRACTION KIT (QIAGEN; Valencia, CA) according to the manufacturer's protocol. These fragments

were cloned into the PCR2.1®–TOPO®TA vector using the PCR2.1®–TOPO®TA CLONING® KIT (INVITROGEN). Bacterial colonies suspected to contain clones of the PCR amplicons were confirmed via restriction enzyme digestion. The DNA sequences of the amplicon fragments were determined using the CEQ™ DYE TERMINATOR CYCLE SEQUENCING KIT according to the manufacturer's instructions, and the sequencing data were analyzed using SEQUENCHER™ version 4.1.4 software (GENE CODES CORP.; Ann Arbor, MI). The resulting sequences produced a 22 kbp contiguous sequence which spanned the entire 15,549 bp fragment containing the 14.8 *KpnI* VirBCDG fragment of pSB1 and the Kanamycin resistance gene, plus both of the approximately 3 kbp flanking *nilA* genomic regions, and extended further into the upstream and downstream *nilA* genomic regions (thereby including LBA4404 chromosomal sequence which was not originally contained in pDAB9698).

The *Agrobacterium tumefaciens* identity of strain DAt16174 was verified via the ketolactose test. Putatively transformed colonies were streaked out on lactose agar and incubated at 28° for 48 hours. The plates were then flooded with Benedict's Solution and monitored at room temperature. Isolates which turned the Benedict's Solution and underlying agar from blue to yellow were thus confirmed to be *Agrobacterium*.

A feature of *A. tumefaciens* strain DAt16174 is that it may be advantageously used as a plant transformation agent for the transfer of T-DNA genes from binary vectors having replication origins of, for example, the IncP, IncW, or VS1 classes. In broad terms, the introduced binary vector may have a replication origin of any class capable of replication in *Agrobacterium* while being compatible with the pTi origin of replication (and associated functions) of the pAL4404 plasmid resident in DAt16174. Thus, it is within the range of possible uses of strain DAt16174 that more than one binary vector plasmid may be co-resident in strain DAt16174 if the plasmids have compatible replication origins (*i.e.*, are of different incompatibility groups). Selection for such introduced binary vectors should not rely on bacterial selectable marker genes conferring either Kanamycin, Rifampicin, or Streptomycin resistance, as the DAt16174 strain is resistant to these three antibiotics.

Binary vectors can replicate autonomously in both *E. coli* and *Agrobacterium* cells. They comprise sequences, framed by the right and left T-DNA border repeat regions, that may include a selectable marker gene functional for the selection of transformed plant cells,

a cloning linker, cloning polylinker, or other sequence which can function as an introduction site for genes destined for plant cell transformation. They can be transformed directly into *Agrobacterium* cells by electroporation, by chemically mediated direct DNA transformation, introduced by bacterial conjugation, or by other methodologies. The *Agrobacterium* used as host cell harbors at least one plasmid carrying a vir region. The vir region is necessary to provide Vir proteins to perform all the requisite functions involved in the transfer of the T-DNA into the plant cell. The plasmid carrying the vir region is commonly a mutated Ti or Ri plasmid (helper plasmid) from which the T-DNA region, including the right and left T-DNA border repeats, have been deleted. Examples of *Agrobacterium* strains that contain helper plasmids and are useful for plant transformation, include, for example, LBA4404, GV3101(pMP90), GV3101(pMP90RK), GV2260, GV3850, EHA101, EHA105, and AGL1. Numerous examples of binary vector systems are reviewed by Hellens *et al.,* (2000, Trends Plant Sci. 5:446–451).

Additionally, the plant transformation advantages conferred upon strain LBA4404(pSB1) [used in the superbinary system] by the pTiBo542-derived *virB* operon (which includes the genes *virB1*, *virB2*, *virB3*, *virB4*, *virB5*, *virB6*, *virB7*, *virB8*, *virB9*, *virB10*, and *virB11*), the *virG* gene, the *virC* operon (which comprises genes *virC1* and *virC2*) and the part of the *virD* operon comprising gene *virD1*, as harbored on the pSB1 plasmid, are retained in strain DAt16174. Because the superbinary *vir* genes listed above are integrated into the LBA4404 chromosome, strain DAt16174 is referred to as a SUPERCHROME strain. In contrast to the superbinary system, use of strain DAt16174 does not require the formation of unstable superbinary plasmids via homologous recombination between pSB1 and shuttle vectors such as pSB11. A further benefit of the SUPERCHROME strain is that standard binary vectors may be introduced into the strain for plant transformation.

EXAMPLE 23: Biochemical and molecular characterization of maize tissues transformed with various *Agrobacterium* strains harboring pDAB101556

A binary plant transformation vector, pDAB101556 (Figure 5), was constructed by a combination of standard cloning methods and GATEWAY™ technology. Binary vector pDAB101556 is based on the IncP-type replication origin of plasmid RK2, and the vector

backbone harbors a bacterial gene conferring resistance to Spectinomycin at 100 µg/mL. The T-DNA border repeats are derived from the TL region of pTi15955. Within the Right Border (RB) and multiple Left Borders (LB) of the T-DNA region of plasmid pDAB101556 are positioned 2 plant-expressible protein coding sequences (CDS). The first gene (selectable marker) comprises the coding region for the AAD1 selectable marker protein (SEQ ID NO:13) (U.S. Patent No. 7,838,733), which is under the transcriptional control of a 1,991 bp maize ubiquitin1 promoter with associated intron1 (U.S. Patent No. 5,510,474). This gene is terminated by a maize Lipase 3'UTR (U.S. Patent No. 7,179,902). The second gene (screenable marker) comprises a CDS for a yellow fluorescent protein (YFP, essentially as disclosed in US Patent No. 7,951,923) transcription of which is controlled by a maize ubiquitin 1 promoter with associated intron 1. This gene is terminated by a maize Per5 3'UTR (U.S. Patent No. 6,384,207).

Plasmid pDAB101556 was successfully introduced by electroporation into cells of *A. tumefaciens* strain LBA4404 to produce strain LBA4404(pDAB101556). This strain/plasmid combination thus comprises a standard binary plant transformation system. Transformants selected by means of resistance to Streptomycin and Spectinomycin were validated by restriction enzyme digestion of plasmid DNA prior to preparation of frozen glycerol stocks and -80° storage. Bulk cells of strain LBA4404(pDAB101556) were harvested from an agar plate inoculated from a frozen glycerol stock and used for maize transformations by methods disclosed in Example 24.

Plasmid pDAB101556 was successfully introduced by electroporation into cells of *A. tumefaciens* strain DAt13192 (see Example 7) to produce strain DAt13192(pDAB101556). This strain/plasmid combination thus comprises a recombination-deficient ternary plant transformation system. Transformants selected by means of resistance to Erythromycin, Kanamycin, and Spectinomycin were validated by restriction enzyme digestion of plasmid DNA prior to preparation of frozen glycerol stocks and storage at -80°. Bulk cells of strain DAt13192(pDAB101556) were harvested from an agar plate inoculated from a frozen glycerol stock and used for maize transformations by methods disclosed in Example 24.

Several attempts were made to introduce DNA of plasmid pDAB101556 into strain DAt20711 (see Example 9), a recombination-proficient ternary system. In all cases,

plasmid pDAB101556 was found to be unstable in this strain and a Dat20711(pDAB101556) strain was not constructed.

Plasmid pDAB101556 was successfully introduced by electroporation into cells of *A. tumefaciens* strain DAt16174 (Example 22) to produce strain DAt16174(pDAB101556). This strain/plasmid combination thus comprises a SUPERCHROME/binary plant transformation system. Transformants selected by means of resistance to Streptomycin, Kanamycin, and Spectinomycin were validated by restriction enzyme digestion of plasmid DNA prior to preparation of frozen glycerol stocks and storage at -80°. Bulk cells of strain DAt16174(pDAB101556) were harvested from an agar plate inoculated from a frozen glycerol stock and used for maize transformations by methods disclosed in Example 24.

EXAMPLE 24: Transformation of maize by *Agrobacterium* strains harboring binary vector pDAB101556

Immature Embryo Production Seeds from a B104 inbred were planted into 3.5 inch SVD pots with METRO MIX 360 (SUN GRO HORTICULTURE Inc.; Bellevue, WA). When the plants reached the V4-V5 growth stage, they were transplanted into 4-gallon pots containing a 1:1 mix of METRO MIX 360 and PROFILE GREENS GRADE calcined clay (PROFILE PRODUCTS LLC; Buffalo Grove, IL), with 20 grams of OSMOCOTE 19-6-12, and 20 grams of IRONITE™ as additives. The plants were grown in a greenhouse using a combination of 1000W HPS (high pressure sodium) and 1000W MH (metal halide) lamps set to a 16:8 light/dark photoperiod if outside light did not exceed 450 W/m^2. In order to obtain immature embryos for transformation, controlled sib or self pollinations were performed.

Immature embryos were isolated at 10 to 13 days post-pollination when embryos were approximately 1.6 to 2.0 mm in size.

Infection and co-cultivation Maize ears were surface sterilized by immersing in 20% commercial bleach with LIQUINOX™ detergent (1 or 2 drops per 500 mL) for 20 minutes and triple-rinsed with sterile water. A suspension of *Agrobacterium* cells containing binary vector pDAB101556 was prepared from bacteria grown on AB solid medium at 20° for 2 to 3 days, followed by growth on YEP solid medium at 28° for 1 to 2 days. Both the AB and YEP media contained appropriate antibiotics supplements as

described in Example 23 for each *Agrobacterium* strain tested with binary vector pDAB101556. Loopfuls of cells scraped from a YEP plate were transferred into 10 to 15 mL of liquid infection medium comprising: MS salts (Frame *et al.*, supra), ISU Modified MS Vitamins (Frame *et al., supra*), 3.3 mg/L Dicamba-ethanol, 68.4 gm/L sucrose, 36 gm/L glucose, 700 mg/L L-proline, pH 5.2, and containing 100-200 µM acetosyringone. The solution was gently pipetted up and down using a sterile 5 mL pipette or vortex mixer until a uniform suspension was achieved, and the concentration was adjusted to an optical density of about 1.0 at 600 nm (OD_{600}) using a Hewlett-Packard P8452a spectrophotometer.

Co-cultivation Immature embryos were isolated directly into a micro centrifuge tube containing 2 mL of the infection medium. The medium was removed and replaced with 1 to 2 mL of fresh infection medium, then replaced with 1.5 mL of the *Agrobacterium* solution. The *Agrobacterium* and embryo solution was incubated for 5 minutes at room temperature and then transferred to co-cultivation medium which contained MS salts, ISU Modified MS Vitamins, 3.3 mg/L Dicamba-ethanol, 30 gm/L sucrose, 700 mg/L L-proline, 100 mg/L myo-inositol, 100 mg/L Casein Enzymatic Hydrolysate, 15 mg/L $AgNO_3$, 100-200 µM acetosyringone, and 2.3 gm/L GELRITE™ (SIGMA-ALDRICH; St. Louis, MO), at pH 5.8. Co-cultivation incubation was for 3 days in the dark at 20°.

Resting and Selection After co-cultivation, the embryos were transferred to a non-selection MS-based resting medium containing MS salts, ISU Modified MS Vitamins, 3.3 mg/L Dicamba-ethanol, 30 gm/L sucrose, 700 mg/L L-proline, 100 mg/L myo-inositol, 100 mg/L Casein Enzymatic Hydrolysate, 15 mg/L $AgNO_3$, 0.5 gm/L MES, 250 mg/L Cefotaxime, and 2.3 gm/L GELRITE™, at pH 5.8. Incubation was continued for 7 days in the dark at 28°. Following the 7 day resting period, the embryos were transferred to Selective Medium. For selection of maize tissues transformed with a superbinary or binary plasmid containing a plant expressible *aad-1* selectable marker gene, the MS-based resting medium (above) was used supplemented with Haloxyfop. The embryos were first transferred to Selection Medium I containing 100 nM Haloxyfop and incubated for 2 weeks, and then transferred to Selection Medium II with 500 nM Haloxyfop and incubated for an additional 2 weeks. Transformed isolates were obtained over the course of approximately 5 weeks at 28° in the dark. If necessary, recovered isolates were bulked up by transferring to fresh Selection Medium II for another 2 weeks before being transferred to

regeneration media.

Those skilled in the art of maize transformation will understand that other methods of selection of transformed plants are available when other plant expressible selectable marker genes (*e.g.* herbicide tolerance genes) are used.

Regeneration I Following the selection process, cultures were transferred to an MS-based Regeneration Medium I containing MS salts, ISU Modified MS Vitamins, 60 gm/L sucrose, 350 mg/L L-proline, 100 mg/L myo-inositol, 50 mg/L Casein Enzymatic Hydrolysate, 1 mg/L $AgNO_3$, 250 mg/L Cefotaxime, 2.5 gm/L GELRITE™ and 500 nM Haloxyfop, at pH 5.8. Incubation was continued for 2 weeks at 28° in the dark.

Regeneration II The cultures were transferred to an MS-based Regeneration Medium II containing MS salts, ISU Modified MS Vitamins, 30 gm/L sucrose, 100 mg/L myo-inositol, 250 mg/L Cefotaxime, 2.5 gm/L GELRITE™, and 500 nM Haloxyfop, at pH 5.8. After 3 weeks at 28° under 16/8 hours photoperiod, with white fluorescent light conditions (approximately 80 $\mu Em^{-2}s^{-1}$), plantlets were excised and transferred to an MS-based or (½ MS-based) shoot/root elongation medium composed of MS salts (or ½ MS salts), ISU Modified MS Vitamins, 0.5 gm/L MES, 30 gm/L sucrose, 100 mg/L myo-inositol, 2.5 gm/L GELRITE™, at pH 5.8. When plantlets reached 4 to 6 cm in length, they were transferred to the growth chamber and eventually to the greenhouse.

Seed production Regenerated plants were transplanted into 3.5 inch SVD pots with METRO MIX 360 and placed in a growth chamber to harden off. When plants reached the V3 growth stage they were moved to the greenhouse, and at the V4/V5 growth stage, they were transplanted into 5-gallon pots containing a 1:1 mix of METRO MIX 360 and PROFILE GREENS GRADE calcined clay, with 20 grams of OSMOCOTE 19-6-12, and 20 grams of IRONITE™ as additives. The plants were grown in the greenhouse under a 16:8 light/dark photoperiod. T1 seed was produced by performing controlled pollinations (backcross to B104). Seed was harvested 6 weeks after pollination.

Multiple maize transformation experiments were performed with engineered *A. tumefaciens* strains LBA4404(pDAB101556), DAt13192(pDAB101556), and DAt16174(pDAB101556), and transgenic calli selected on inhibitory concentrations of Haloxyfop were carried forward for plantlet regeneration and further studies. In total, 16 events were retained in the LBA4404(pDAB101556) transformations, 49 events were

retained in the DAt13192(pDAB101556) transformations, and 60 events were retained in the DAt16174(pDAB101556) transformations (Table 7).

Copy numbers of the *aad-1* transgene in transgenic T0 plants were estimated by hydrolysis probe assays ((Bubner and Baldwin, *supra*) using gene-specific oligonucleotides. Southern blot analyses of *NcoI*-cleaved DNA prepared from the selected events by a cetyl trimethylammonium bromide extraction method were performed using a PCR amplified fragment of the *aad-1* gene as 32P-labeled probe. Further, the presence of integrated backbone vector sequences originating from pDAB101556 was detected by hydrolysis probe analyses.

Thus, strain DAt16174, a SUPERCHROME strain comprising a full set of pTiACH5-derived *vir* genes harbored on pAL4404, and further comprising a partial set of pTiBo542-derived *vir* genes integrated into the LBA4404 chromosome at the *nilA* locus, is able to efficiently produce transformed maize plants. Further, while having a somewhat lower overall transformation efficiency than that obtained with the ternary strain, the quality of SUPERCHROME-produced events is superior, with 90% of the events produced having single copy inserts with no detectable backbone contamination.

While this invention has been described in certain example embodiments, which are intended as illustrative of a few aspects of the invention, the present invention may be further modified within the spirit and scope of this disclosure. This application is therefore intended to cover any variations, uses, or adaptations of the invention using its general principles. Further, this application is intended to cover such departures from the present disclosure as come within known or customary practice in the art to which this invention pertains and which fall within the limits of the appended claims.

All references, including publications, patents, and patent applications, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein. The references discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

Table 1. Representative incompatibility groups and some example plasmids that are classified as belonging to these incompatibility groups.

Incompatibility Group	Plasmids
FI	F, R386
FII	R1
FIII	Col B-K99, Col B-K166
FIV	R124
I	R62, R64, R483 (at least 5 subgroups)
J	R391
N	R46
O	R724
P	RP4, RK2
Q	RSF1010
T	R401
W	R388, S-a

Table 2. Summary of *in vitro* bioassay results.

Construct	Mean CEW Score (96 well assay)	Mean FAW Score (96 well assay)	Mean ECB % Damage (32 well assay)
Negative Control	1.64	1.78	75.9
101513	0.59	0.88	17.4
101514	0.80	0.78	12.0

Table 3. Production (in ppm; parts per million) of the AAD1, Cry1Ca, Cry1Fa, and Cry1Ab proteins in maize plants transformed with binary vector pDAB101513.

Event Name	AAD1	Cry1Ca	Cry1Fa	Cry1Ab
101513[37]-008.002	830	370	160	84
101513[37]-020.001	550	410	210	100
101513[39]-011.002	380	270	150	27
101513[44]-031.003	740	300	100	40
101513[45]-022.002	380	270	86	32
101513[45]-023.001	300	340	160	31
101513[49]-040.002	220	270	170	21
101513[49]-040.003	210	410	220	26
101513[49]-041.001	340	270	200	21

Table 4. Results of maize transformation experiments with strains of *A. tumefaciens* harboring plasmid pDAB101513.

A	B	C	D	E	F	G	H
Strain	Embryos Treated	Regenerable events (% X-form. efficiency)	T_0 Events Analyzed	Events with all 4 genes (%)	Low-Copy Events with all 4 genes (%)	Events of Col. F producing all 4 proteins (%)	Events of Col. G active against all three pests (%)
EHA105	2469	6 (0.24)	4	1 (25)	1 (25)	0 (0)	0 (0)
DA2552	630	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)
DAt13192	1945	34 (1.75)	25	21 (84)	14 (56)	9 (64)	9 (100)

Table 5. Results of maize transformation experiments with strains of *A. tumefaciens* harboring plasmid pDAB101514.

A	B	C	D	E	F	G	H
Strain	Embryos Treated	Regenerable events (% X-form. efficiency)	T0 Events Analyzed	Events with all 4 genes (%)	Low-Copy Events with all 4 genes (%)	Events of Col. F producing all 4 proteins (%)	Events of Col. G active against all three pests (%)
EHA105	3499	11 (0.31)	11	2 (18)	1 (50)	0 (0)	0 (0)
DA2552	771	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)
DAt13192	926	17 (1.83)	15	12 (80)	9 (75)	8 (89)	6 (75)

Table 6: PCR primers used for molecular confirmation of integration of the 15,549 bp fragment containing the 14.8 *KpnI* VirBCDG fragment of pSB1 and Kanamycin resistance gene into the *nilA* genomic region of the *Agrobacterium tumefaciens* strain DAt16174 chromosome.

PrimerPair	SEQ ID NO:	Primer Name	Primer Sequence (5' to 3')	Amplicon Size
1	SEQ ID NO:26	H3-2 Down	ATCTTACCTTCCTTTTCGTTTTCCAAC	4,248 bp
	SEQ ID NO:27	Set2 5'	CTGCTTGGATGCCCGAGGCATAGAC	
2	SEQ ID NO:28	Vir Screen 1 5'	CATCCAAGCAGCAAGCGCGTTACG	7,696 bp
	SEQ ID NO:29	Vir Screen 4 3'	GTCTATGCCTCGGGCATCCAAGCAG	
3	SEQ ID NO:30	Vir Screen 5 5'	GAGACCGTAGGTGATAAGTTGCCC	6,917 bp
	SEQ ID NO:31	Vir Screen 8 3'	TCTCATTTAGGGGCTGGCTCCAAC	
4	SEQ ID NO:32	VirG	TGCGAGCAACATGGTCAAACTCAG	3,650 bp
	SEQ ID NO:33	VirB 1 3'	GACATGCAGAACAACGAGAAACGA	
5	SEQ ID NO:34	PSB1-1 5'	GCACACCGAAATGCTTGGTGTAGA	4,126 bp
	SEQ ID NO:35	*nilA* For1	GGCCGTGCACGGCATCAATCTCGAA	

5

Table 7. Analyses of transgenic events produced by three *Agrobacterium* strains harboring plasmid pDAB101556.

Agrobacterium System	Total Events With Inserts	Transformation Frequency (%)	% With Single-Copy Inserts	% With Single-Copy Inserts, and Backbone-Free	No. With Single-Copy Inserts, and Backbone-Free
Ternary DAt13192(pDAB101556)	60	8.5	33	75	15
SUPERCHROME DAt16174(pDAB101556)	49	6	43	90	19
Binary LBA4404(pDAB101556)	16	3	88	86	14

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CLAIMS

What is claimed is:

1. A method for transforming a plant, comprising contacting a cell of the plant with an *Agrobacterium* strain having at least one pTi helper plasmid comprising a 14.8 *KpnI* fragment of pSB1 and a pTi plasmid having at least one disarmed T-DNA region, the T-DNA region comprising at least a right T-DNA border and exogenous DNA adjacent to the border, wherein the plasmids have differing origins of replication relative to each other.

2. The method of claim 1, wherein the 14.8 *KpnI* VirBCDG fragment isolated from pSB1 in *Agrobacterium* strains has a deficiency in RecA function.

3. A method for transforming a plant, comprising contacting a cell of the plant with a bacterium of the genus Agrobacterium having a 14.8 KpnI VirBCDG fragment of pSB1 and a pTi plasmid having at least one disarmed T-DNA region, wherein the 14.8 KpnI VirBCDG fragment has been integrated into a neutral integration site of a chromosome of the bacterium.

4. The method for transforming a plant according to any of claims 1-2, wherein the bacterium further comprises a plasmid having a T-DNA region adjacent to at least one *Agrobacterium* T-DNA border, the plasmid having a replication origin of an IncP incompatibility group.

5. The method for transforming a plant according to claim 3, wherein the bacterium further comprises a plasmid having a T-DNA region adjacent to at least one *Agrobacterium* T-DNA border.

6. The method for transforming a plant according to claim 3, wherein the *Agrobacterium* strain is deficient in RecA functionality.

7. The method for transforming a plant according to any of claims 4-6, wherein the T-DNA region contains three or more gene sequences.

8. The method for transforming a plant according to any of claims 4-7, wherein the T-DNA region contains equal to or greater than 25,000 nucleotide base pairs.

9. The method for transforming a plant according to any of claims 4-8, wherein the T-DNA region is inserted into a single location in the plant cell when the plant is transformed.

10. The method for transforming a plant according to any of claims 4-9, wherein the T-DNA region comprises more than one gene sequence and the gene sequences have equal to or greater than 60% sequence homology.

11. The method for transforming a plant according to any of claims 4-10, wherein the T-DNA region encodes one or more of an insecticidal protein, a herbicidal protein, or a mixture of insecticidal proteins and herbicide tolerance proteins.

12. The method for transforming a plant according to any of claims 4-11, wherein the T-DNA region encodes a Cry1Ca insecticidal protein, a Cry1F insecticidal protein, and a Cry1Ab1 insecticidal protein.

13. The method for transforming a plant according to any of claims 4-12, wherein the T-DNA region encodes a Cry1Ca insecticidal protein, a Cry1F insecticidal protein, a Cry1Ab1 insecticidal protein, and an AAD-1 herbicide tolerance protein.

14. The method according to any of claims 1-13, wherein the plant is a monocot.

15. The method according to any of claims 1-14, wherein the 14.8 *KpnI* VirBCDG fragment is cloned into the *Kpn I* site of a pDAB9291plasmid.

16. The method according to any of claims 1-15, wherein the pTi helper plasmid is plasmid pMP90.

17. The method according to any of claims 1-16, wherein the pTi helper plasmid is plasmid pTiC58Δ.

18. The method according to claim 15, further comprising transforming the *Agrobacterium* strain using plasmid pDAB9292 DNA.

19. The method according to any of claims 1-18, further comprising a step of selecting a transformed cell or a transformed tissue, after subjecting said cultured tissue to transformation.

20. An *Agrobacterium* strain having at least one pTi helper plasmid comprising a 14.8 *KpnI* fragment of pSB1 and a pTi plasmid having at least one disarmed T-DNA region, wherein the plasmids have differing origins of replication relative to each other.

21. The *Agrobacterium* strain of claim 20, wherein the *Agrobacterium* strain has a deficiency in RecA function.

22. An *Agrobacterium* strain having transformation-enhancing properties comprising a 14.8 *KpnI* VirBCDG fragment isolated from pSB1 and a pTi plasmid having at least one disarmed T-DNA region.

23. The *Agrobacterium* strain according to any of claims 20-21, wherein the bacterium further comprises a plasmid having a T-DNA region adjacent to at least one *Agrobacterium* T-DNA border.

24. The *Agrobacterium* strain according to claim 22, wherein the bacterium further comprises a plasmid having a T-DNA region adjacent to at least one *Agrobacterium* T-DNA border.

25. The *Agrobacterium* strain according to claim 24, wherein the *Agrobacterium* strain is deficient in RecA functionality.

26. The *Agrobacterium* strain according to any of claims 23-25, wherein the T-DNA region contains three or more gene sequences.

27. The *Agrobacterium* strain according to any of claims 23-26, wherein the T-DNA region contains equal to or greater than 25,000 nucleotides.

28. The *Agrobacterium* strain according to any of claims 23-27, wherein the T-DNA region comprises more than one gene sequence and the gene sequences have greater than 60% sequence homology.

29. The *Agrobacterium* strain according to any of claims 23-28, wherein T-DNA region encodes one or more of an insecticidal protein, a herbicidal proteins, or a mixture of insecticidal proteins and herbicide tolerance proteins.

30. The *Agrobacterium* strain according to any of claims 23-29, wherein the T-DNA region encodes a Cry1Ca insecticidal protein, a Cry1F insecticidal protein, and a Cry1Ab1 insecticidal protein.

31. The *Agrobacterium* strain according to any of claims 23-30, wherein the T-DNA region encodes a Cry1Ca insecticidal protein, a Cry1F insecticidal protein, a Cry1Ab1 insecticidal protein, and an AAD-1 herbicide tolerance protein.

32. A *nilA* genomic locus of *Agrobacterium tumefaciens*, wherein a polynucleotide sequence is integrated into the *nilA* genomic locus.

33. The *nilA* genomic locus of claim 32, wherein the polynucleotide sequence comprises a *vir* gene.

34. An *Agrobacterium* strain with a 14.8 *KpnI* VirBCDG fragment of SB1 integrated into a neutral integration site on the *Agrobacterium* chromosome.

35. The *Agrobacterium* strain according to claim 34, wherein the neutral integration site is a *nilA* genomic locus.

36. The *Agrobacterium* strain according to any of claims 34-35, wherein the *Agrobacterium* strain is deficient in RecA functionality.

37. The *Agrobacterium* strain according to any of claims 34-36, wherein the *Agrobacterium* strain is *Agrobacterium tumefaciens*.

38. An *Agrobacterium* strain LB4404 comprising a 14.8 *KpnI* VirBCDG fragment of pSB1on a pTi helper plasmid and a pTi plasmid having at least one disarmed T-DNA region and has exogenous DNA adjacent to at least one *Agrobacterium* T-DNA border, wherein the plasmids have differing origins of replication relative to each other.

39. A plant according to any of claims 1-19.

40. The plant according to claim 39, wherein any genetic traits introduced to the plant by the transformation are stably produced in progeny of the plant.

41. A plant according to any of claims 4-13, wherein the T-DNA region is stably incorporated into the plant DNA.

42. The plant according to claim 41, wherein any genes encoded by the T-DNA region are expressed in the plant.

43. The plant according to any of claims 41-42, wherein any genes encoded by the T-DNA region are stably produced in progeny of the plant.

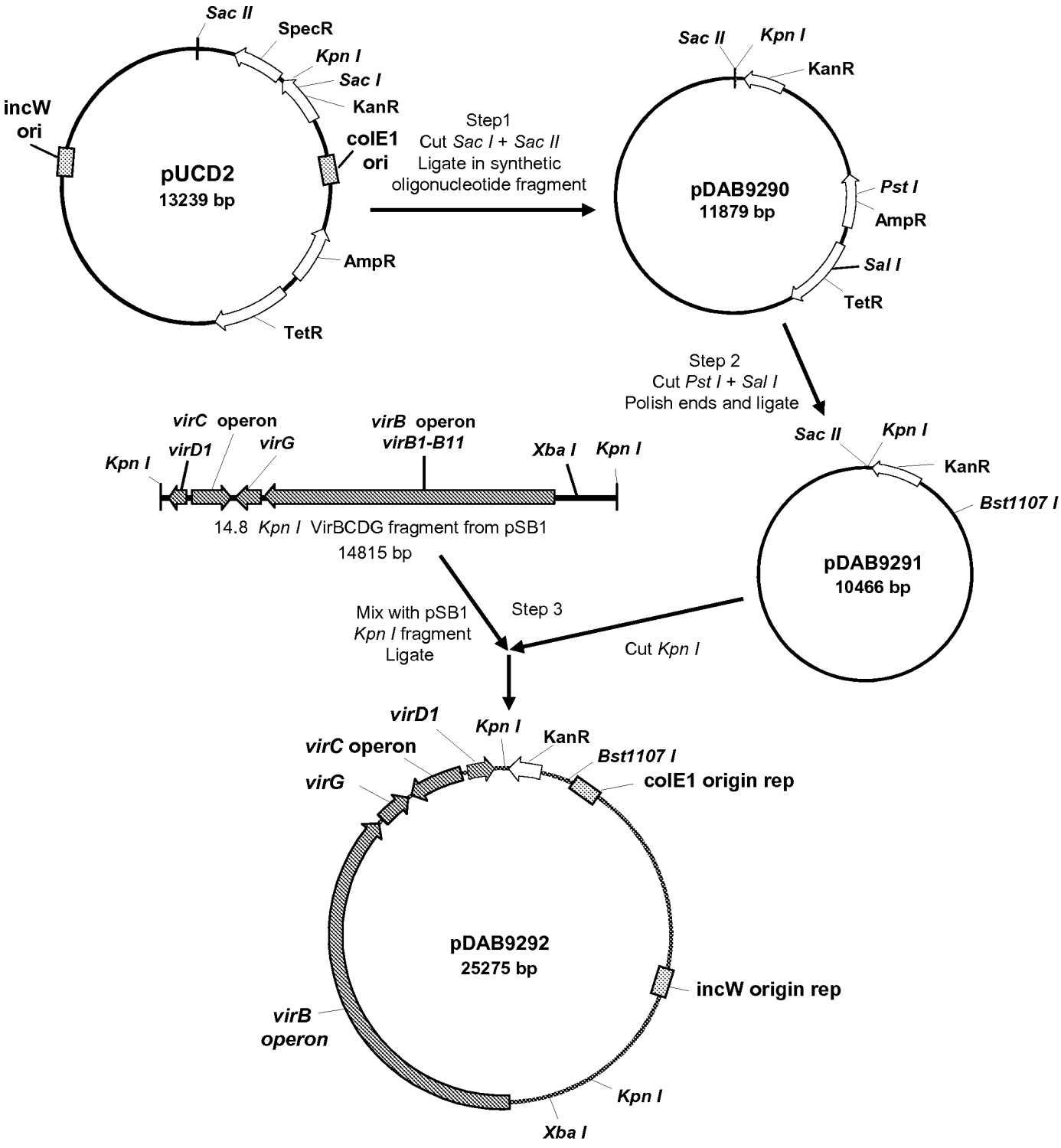
44. The plant according to claim 41, wherein the plant stably expresses Cry1Ca insecticidal proteins, Cry1F insecticidal proteins, Cry1Ab1 insecticidal proteins, and AAD1 herbicidal proteins.

45. The plant according to claim 44, wherein the plant is maize.

46. *Agrobacterium* strain LBA4404 comprising at least one vir gene from a 14.8 *KpnI* VirBCDG fragment isolated from pSB1 integrated into a neutral integration site on the *Agrobacterium* chromosome.

47. A fertile transgenic corn plant, or progeny thereof, which expresses insecticidal amounts of Cry1Ca protein, Cry1F insecticidal protein, Cry1Ab1 insecticidal protein, and herbicide-tolerant amounts of AAD-1 protein, wherein the Cry1Ca, Cry1F, Cry1Ab1, and AAD1 proteins are collectively expressed from a single locus of recombinant DNA stably incorporated in the genome of the plant.

48. The fertile transgenic corn plant of claim 47, wherein the single locus of recombinant DNA is substantially free of vector backbone sequences from a pTi DNA plasmid.



Oligonucleotide for Step 1

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--GGGGTACCCGCTACCCGGGTCATGATGTCTAACGTTTGACATGAGGGGCGGCCAAGGGCGCCAGCCCTTGGAC
CGCCCCATGGGCGATGGGCCCAGTACTACAGATTGCAAACTGTACTCCCCGCCGGTTCCCGCGGTCGGGAACCTG

GTCCCCCTCGATGGAAGGGTTAGGCATCACTGCGTGTTCGCTCGAATGCCTGGCGTGTTTGAACCATGTACACGG
CAGGGGGAGCTACCTTCCCAATCCGTAGTGACGCACAAGCGAGCTTACGGACCGCACAAACTTGGTACATGTGCC

CTGGACCATCTGGGGTGGTTACAGTACCTTGCCTCTCAAACCCCGCTTTCTCGTAGCATCGGATCGCTCGCAAGT
GACCTGGTAGACCCCACCAATGTCATGGAACGGAGAGTTTGGGGCGAAAGAGCATCGTAGCCTAGCGAGCGTTCA

TGCTCGGCGACGGGTCCGTTTGGATCTTGGTGACTTCGGGATCATTGAACAGCAACTCAACCAGAGCT
ACGAGCCGCTGCCCAGGCAAACCTAGAACCACTGAAGCCCTAGTAACTTGTCGTTGAGTTGGTC----
```

Fig. 1

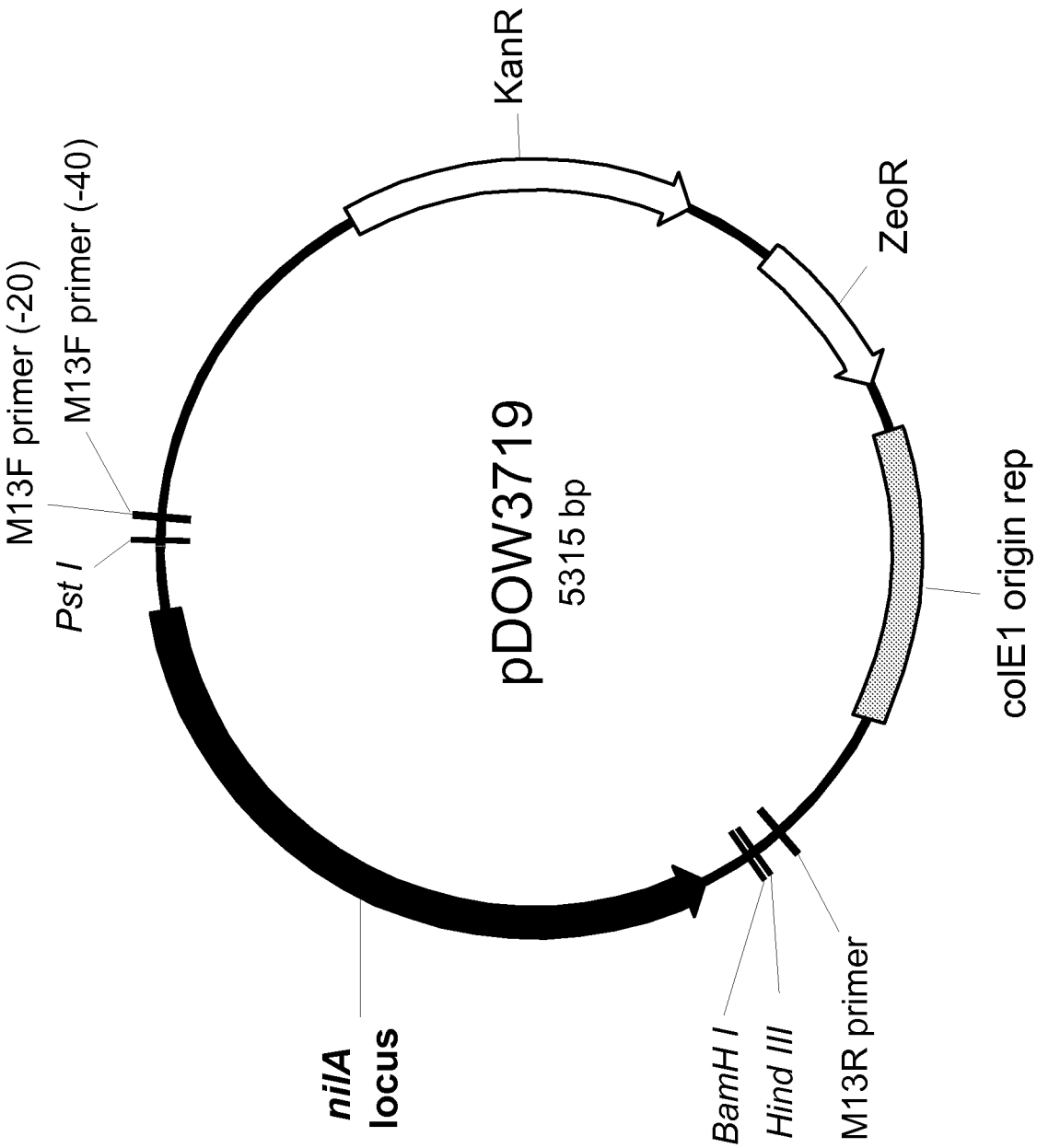
pDOW3719
5315 bp
KanR
ZeoR
colE1 origin rep
M13R primer
BamH I
Hind III
nilA locus
M13F primer (-20)
M13F primer (-40)
Pst I

Fig. 2

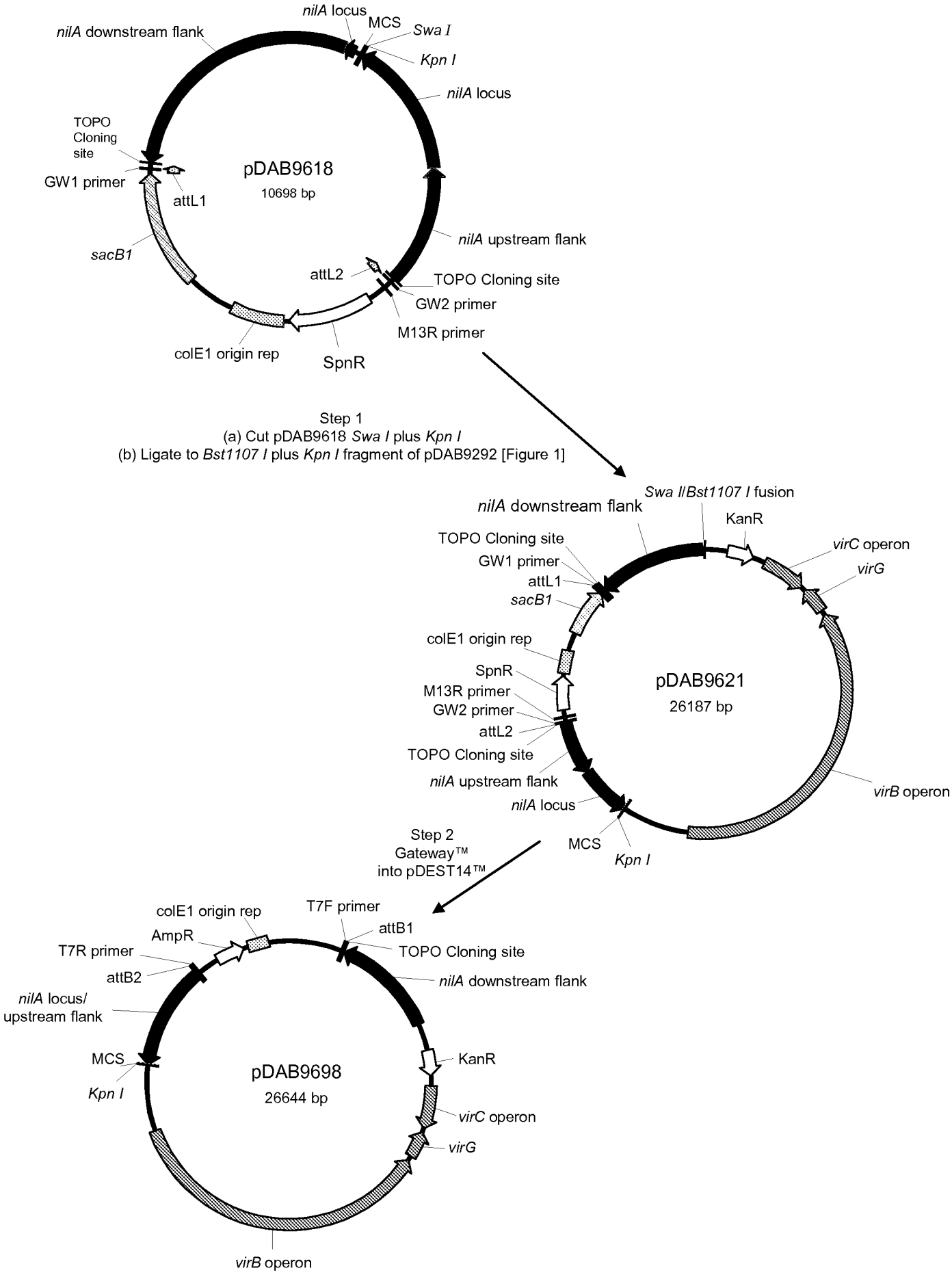
nilA locus
MCS
Swa I
Kpn I
nilA locus
nilA downstream flank
TOPO Cloning site
GW1 primer
attL1
pDAB9618
10698 bp
sacB1
attL2
TOPO Cloning site
GW2 primer
M13R primer
nilA upstream flank
colE1 origin rep
SpnR
Step 1
(a) Cut pDAB9618 Swa I plus Kpn I
(b) Ligate to Bst1107 I plus Kpn I fragment of pDAB9292 [Figure 1]
Swa I/Bst1107 I fusion
nilA downstream flank
KanR
TOPO Cloning site
virC operon
GW1 primer
virG
attL1
sacB1
colE1 origin rep
SpnR
M13R primer
pDAB9621
26187 bp
GW2 primer
attL2
TOPO Cloning site
nilA upstream flank
virB operon
nilA locus
MCS
Kpn I
Step 2
Gateway™
into pDEST14™
colE1 origin rep
T7F primer
AmpR
attB1
T7R primer
TOPO Cloning site
attB2
nilA downstream flank
nilA locus/
upstream flank
MCS
KanR
pDAB9698
26644 bp
Kpn I
virC operon
virG
virB operon

Fig. 3

Fig. A

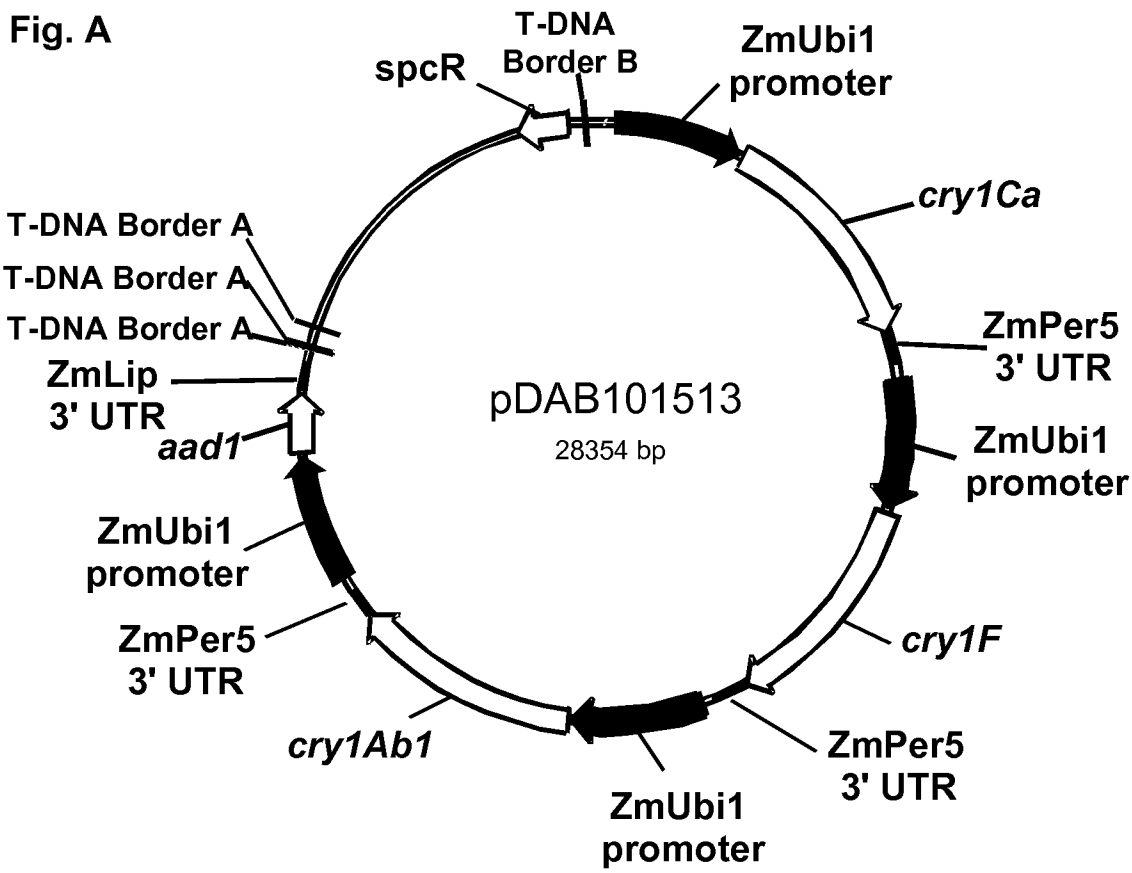

Fig. B

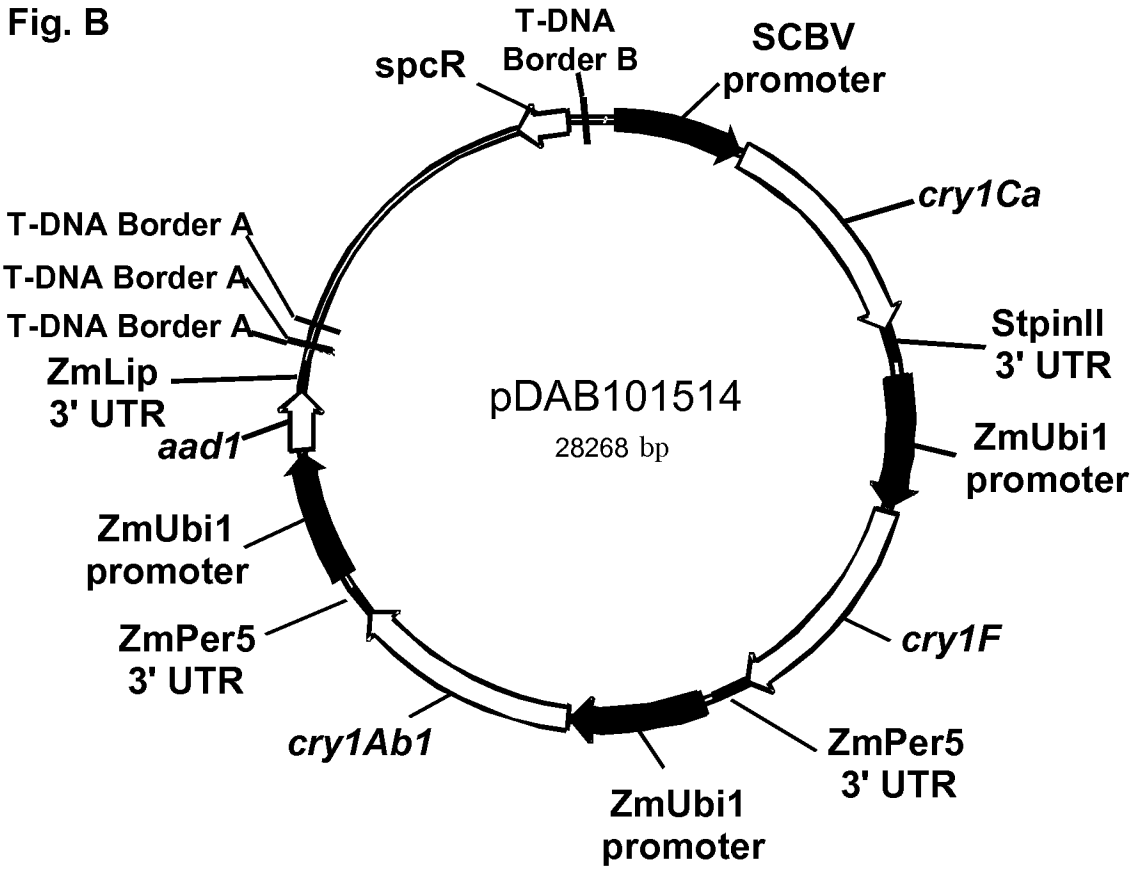

Fig. 4

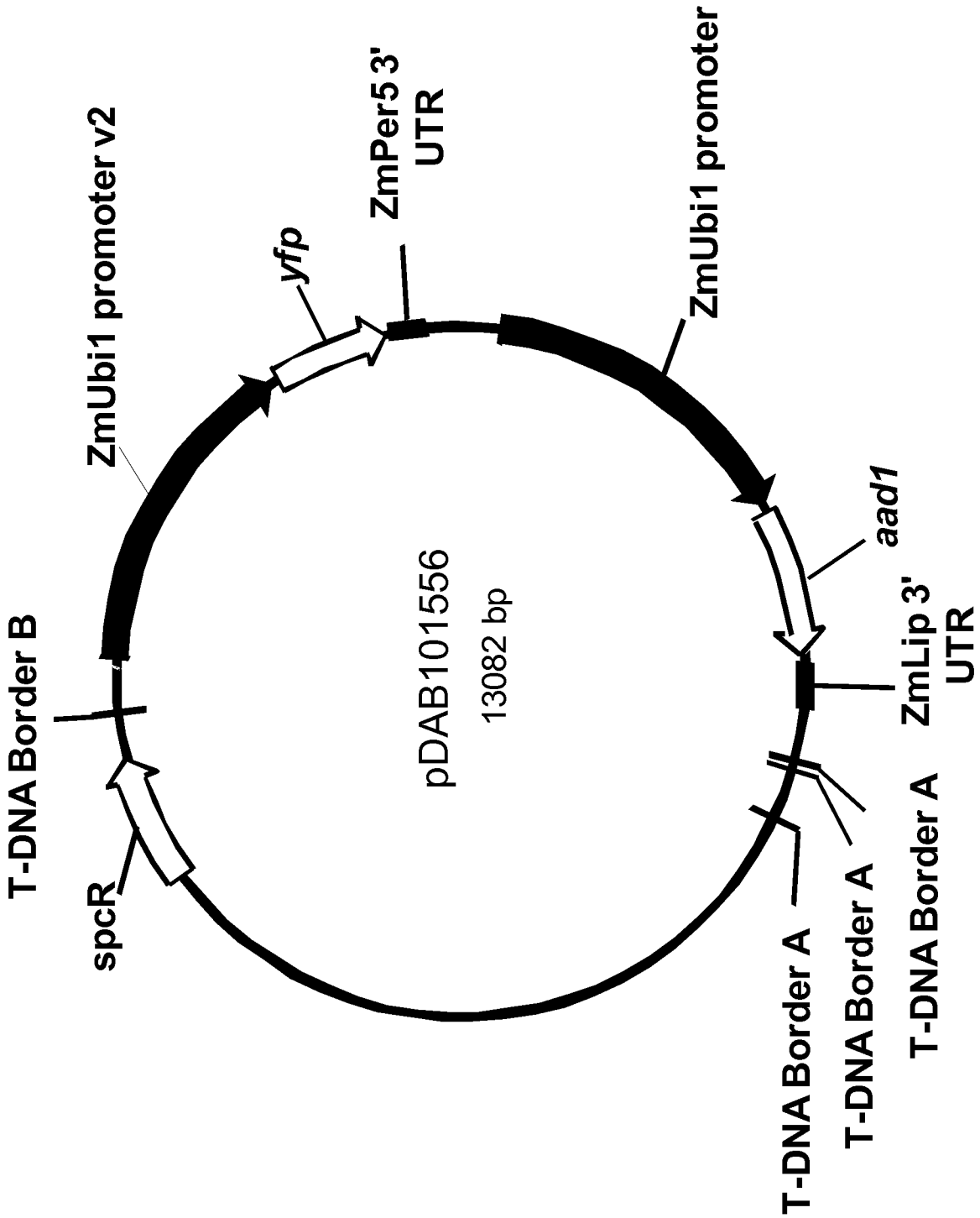
T-DNA Border B
ZmUbi1 promoter v2
yfp
ZmPer5 3' UTR
ZmUbi1 promoter
aad1
ZmLip 3' UTR
T-DNA Border A
T-DNA Border A
T-DNA Border A
spcR
pDAB101556
13082 bp

Fig. 5